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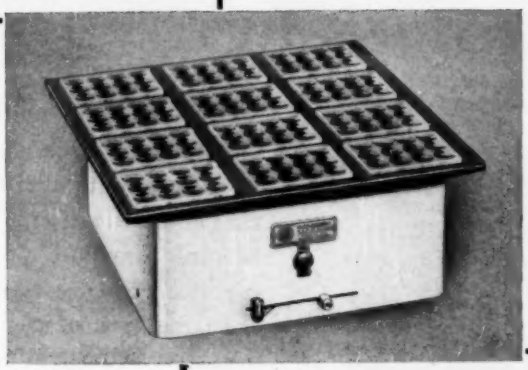

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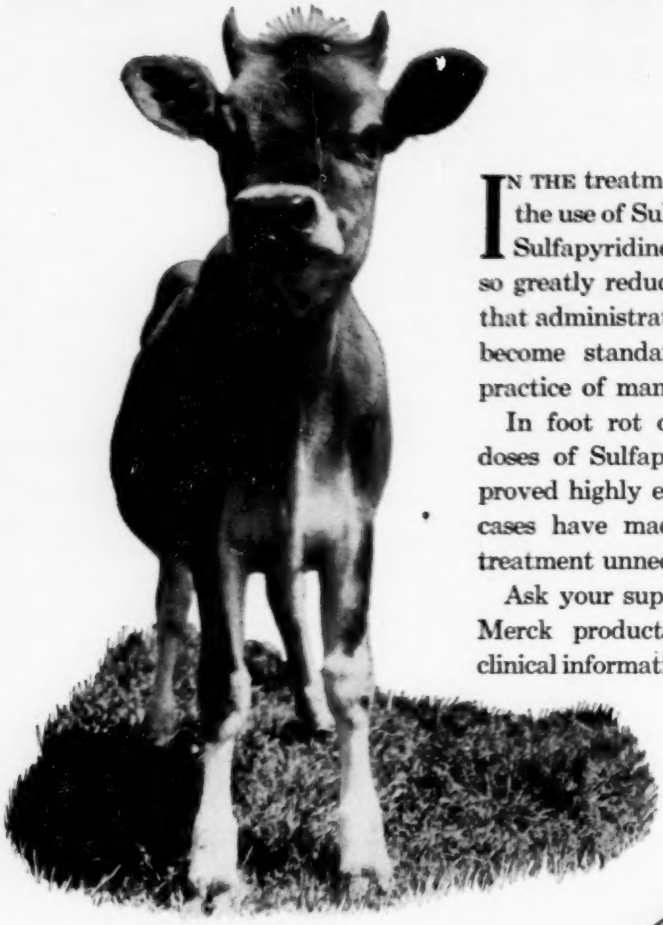

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Pathogenicity of *Salmonella Choleraesuis:* Maintaining Virulence by Lyophilization

H. W. SCHOENING, V.M.D., C. N. DALE, D.V.M., L. O. MOTT, D.V.M., and R. T. HABERMANN, D.V.M.

Washington, D. C.

LITERATURE CITED

THE FIRST report on the pathogenicity of *Salmonella choleraesuis* (*Bacterium suipestifer*) was made by Salmon[1] when, in 1885, he reported that this organism was the cause of hog cholera. Following the findings of Dorset, Bolton, and McBryde[2] that hog cholera was caused by a filterable virus, *S. choleraesuis* (*Bact. suipestifer*) was considered as only a secondary invader until Glässer[3] in 1907, and Dammann and Stedefeder[4] in 1910, showed that paratyphoid infection occurred in swine in the absence of hog-cholera virus and was caused by *S. choleraesuis.* In 1927, Murray, Biester, Purwin, and McNutt[5] concluded that *S. choleraesuis* was the primary cause of infectious enteritis of swine (so-called necrotic enteritis).

In 1928, Biester, Murray, McNutt, and Purwin[6] stated that "in our early studies[5] about 20 cc. of a given culture, after frequent animal passages, would prove an infective dose. When the same culture was kept *in vitro,* its virulence was diminished to such an extent that 150 cc. would neither kill nor produce advanced intestinal lesions." Since they planned to produce fatal cases for study, they used 250 cc. of a culture which had been kept *in vitro* a long time and subjected to only two animal passages. In the discussion which followed the presentation of the paper, this question was asked: "Is this disease contagious, and have you attempted exposure work on it?" The reply: "It is an infectious disease. After these pigs have been fed a culture of virulent *S. suipestifer,* they develop the disease. As to being contagious, it seems to be one of those diseases which, when one wants to produce it experimentally by contact, it cannot always be done. *There seems to be a slight resistance or some* aiding, disease-producing factor. A small amount of a typhoid culture fed to a human being would produce disease, but in swine the condition produced by *S. suipestifer* seems to be more or less of a relative disease." Since this publication, many reports[7-29] have recorded observations on the pathogenicity of *S. choleraesuis.* Many of these reports have raised the question of the degree of virulence and infectivity of various strains used in experimental work.

In 1940, Davis, Freeman, and Madsen[30] reported on some nutritional experiments with swine and suggested "that infectious necrotic enteritis is a secondary complication caused by intestinal invasion of the organisms *S. choleraesuis,* and probably other organisms, after the symptoms of a deficiency of nicotinic acid have developed in the pig."

The results of experiments on the maintenance of the virulence and infectivity of cultures of *S. choleraesuis* by the process of lyophilization and the incidence of lesions of a necrotic enteritis which they produced in swine are given in this paper.

EXPERIMENTAL

Source of Cultures.—In the work that follows, two cultures, designated 4623L and 5812L, are considered as constants.

Culture 4623 originated from a herd of swine in which a number of animals were succumbing to an acute disease. Three dead pigs were brought to the laboratory for examination. *Salmonella choleraesuis** was obtained from the heart blood, liver, spleen, kidney, and lung of 1 pig and from only the liver of the second pig. It was not obtained from any of the tissues of the third pig. Viscera from these 3 pigs were fed to 2 cholera-susceptible pigs and four days later a cholera-

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We express our appreciation to Dr. L. T. Giltner, Pathological Division, for helpful suggestions in these studies and to Dr. P. R. Edwards, University of Kentucky, Lexington, for the serologic identifications of the various types of Salmonella that were used. The chemical analyses of the feeds used in experiment 6 for nicotinic acid content were made by Mr. W. C. Powick, Animal Husbandry Division.

*Cultures designated 4623L, 5812L, and Howard were identified serologically as *S. choleraesuis* var. *kunzendorf* by Dr. P. R. Edwards. The other cultures of *S. choleraesuis* were identified as the same type by their biochemical characteristics. The other types of Salmonella were also identified serologically by Dr. Edwards.

susceptible pig was added as a contact. Two days later, the 2 pigs that were fed viscera were moribund and were destroyed. The following day, viscera from these 2 pigs were fed to a normal cholera-susceptible pig. It died seven days later. Four days later, viscera from this pig were fed to 2 normal cholera-susceptible pigs. They died seven days later. The contact pig died on the ninth day. *Salmonella choleraesuis* was recovered from all the tissues of these 6 cholera-susceptible pigs.

Eighteen days after the sixth pig died, a cholera-susceptible pig was injected intravenously with 21 cc. of pooled, 24-hour broth culture of the six strains obtained from the 6 pigs and the strain obtained from 1 of the 3 original pigs. This pig was moribund on the second day. It was destroyed by bleeding. The blood was allowed to clot, and on the following day, a cholera-susceptible pig (4601) was injected intravenously with 15 cc. of the unfiltered serum. This pig died nine days later. *Salmonella choleraesuis* (4601) was obtained from the tissues. Eleven days later, a cholera-immune pig (4623) was injected intravenously with 17 cc. of a 24-hour broth culture of *S. choleraesuis* obtained from pig 4601. Pig 4623 died three days later. *Salmonella choleraesuis* was obtained from the tissues. Eight days later agar slant cultures were submitted to the Public Health Service Laboratory for preservation.

Cultures 4623L and 5812L have been preserved by the Flosdorf-Mudd lyophile apparatus.[21] The procedure consists of suspending the 24-hour bacterial growth from agar slants in skimmilk. The suspension is distributed in 2-cc. glass ampules, ½ cc. to each ampule. The suspensions are rapidly frozen at a very low temperature. The ampules are then attached to apparatus for rapid dehydration under high vacuum. After eighteen to twenty-four hours' dehydration, the ampules are sealed under vacuum. The original 4623 culture was preserved on April 6, 1937, eight days after isolation as mentioned above, by a similar procedure in the U. S. Public Health Service Laboratory by Dr. M. Pitman, Washington, D. C., at the request of Dr. W. S. Gochenour.*

Fourteen months after the original culture was preserved, an ampule was opened, and after agar slants were seeded, milk suspensions of 24-hour growths from these slants were pooled and lyophilized (L) as described above and the culture identified as 4623L (5/29/38).

On Jan. 20, 1942, an ampule of 4623L (5/29/38) was opened and another batch of culture was lyophilized and identified as 4623L (1/21/42).

Five months later, an ampule of 4623L (1/21/42) was opened and a 24-hour broth culture prepared. A pig was injected intravenously with this culture. After death, the heart blood was cultured, and the culture of *S. choleraesuis* obtained was again injected intravenously into a pig. Six such passages were made in series. In the sixth case (pig 5812), the heart blood was seeded on a number of agar slants and the 24-hour growths suspended in milk, pooled, and lyophilized. This culture was identified as 5812L (7/10/42).

Strains O, P, and Howard were isolated from the heart blood or spleen of pigs that had died in hog-cholera "breaks." The other types, except *Salmonella enteritidis*, were isolated from colon contents or feces of swine. The interval of time between their isolation and injection was twenty months for *Salmonella california*, eighteen months for *Salmonella meleagridis*, twelve months for *Salmonella oranienberg* and *S. enteritidis*, eight months for *Salmonella poona*, and seven months for *Salmonella senftenberg*, *Salmonella worthington*, and *Salmonella derby*. *Salmonella enteritidis* was isolated from the heart blood of a mouse.

Intravenous Injections of Broth Cultures.—Of 74 pigs that were injected intravenously at various times over a period of five years with 10 to 25 cc. of 24-hour broth cultures of Salmonella, 61 were injected with *S. choleraesuis;* 1 with *S. senftenberg*, *S. worthington*, and *S. derby*, respectively; and 2 with *S. california*, *S. oranienberg*, *S. meleagridis*, *S. enteritidis*, and *S. poona*, respectively. Forty-six of the 74 pigs injected were normal, weighing 60 to 250 lb., and had had no artificial exposure to *S. choleraesuis*. The other 28 pigs, weighing 200 to 300 lb., had been used previously in *S. choleraesuis* experiments and may have developed some immunity from exposure by contact, subcutaneous injections, or feeding.

For the 46 normal pigs, the methods of injecting and the results obtained are shown in table 1. All of the 26 pigs injected with *S. choleraesuis* 4623L, or strains that had been isolated from the heart blood or spleen within a seven-day period previous to injection, died within seventy-two hours. When the strains of *S. choleraesuis* used had been isolated from the heart blood or spleen for longer periods, only 3 of 17 pigs died within seventy-two hours. The pigs injected with *S. senftenberg*, *S. worthington*, and *S. derby* showed some fever but were not severely affected. The failure of these pigs to be more severely affected by these types of Salmonella may have been due to the swine being less susceptible to these types or to loss of virulence of the culture during the seven months' interval between isolation and injection.

Of the 28 pigs that may have had some im-

*Dr. Gochenour resigned from the Pathological Division in November, 1938.

munity to *S. choleraesuis* infection, 18 were injected with various strains of *S. choleraesuis* that had been isolated from one week to five months previously. Nine died, 3 on the second day, 2 on the fourth day, 1 on the fifth day, 2 on the sixth day, and 1 on the sixteenth day following exposure. Three had severe reactions; 1 was destroyed on the fourteenth day and 2 on the thirty-third day, when moribund. One had a severe reaction but had recovered by the eleventh day. One had a slight reaction and had recovered by the seventeenth day except for slight stiffness. Four had slight reactions and had recovered by the fifth day.

No reaction, except for some fever on the first day following the injection, occurred in 2 pigs injected with *S. california*, 2 pigs injected with *S. meleagridis*, and 2 pigs injected with *S. oranienberg*. One of 2 pigs injected with *S. poona* died on the third day. The other pig had a severe reaction but recovered by the fourteenth day. Two pigs injected with *S. enteritidis* had severe reactions; one recovering by the fourteenth day when the other one was moribund and destroyed.

Failure to obtain as high a mortality with *S. choleraesuis* in this group of pigs as was obtained in the group of normal pigs may have been due to the following: less virulent cultures, a difference in the degree of immunity which the animals may have possessed, unknown factors increasing resistance, or some combination of these.

Feeding Broth Cultures.—In order to show the virulence of the two strains, 4623L and 5812L, over a period of years, seven experiments are reported here in which broth cultures of one or both of these strains were fed to some of the pigs in each experiment. In several of the experiments, other pigs were fed broth cultures of other strains for comparative purposes.

Experiment 1.—In this experiment, 4 or 5 pigs raised at the Bureau's Animal Disease Station at Beltsville, Md., and weighing from 70 to 90 lb., were fed 24-hour broth culture of 4623L (5/29/38). These 5 pigs were the control group in an experiment in which an attempt was made to produce a hog-cholera break. On Oct. 31, 1940, 3 of these 5 pigs were fed 25 cc. each, and a fourth pig was fed 50 cc. One of the 3 pigs fed 25 cc. was also fed 25 cc. on the following day, and another was fed 25 cc. on each of two following days. Four days after the last feeding of this pig, the fifth pig was placed in contact with it. The 4 pigs that were fed culture had fever and impaired appetites by the third day after the initial feeding. The pig fed the single dose of 25 cc. recovered by the fourteenth day. It was killed on the twentieth day and necropsy revealed a slight necrotic enteritis. The pig fed two, 25-cc. doses had recovered by the seventeenth day. The intestine was normal when the animal was destroyed on the forty-first day after the initial feeding. The pig fed three, 25-cc. doses became chronically unthrifty and died on the forty-second day. Necropsy revealed a marked necrotic enteritis. The pig fed 50 cc. was killed on the seventh day and necropsy revealed a marked necrotic enteritis. The contact pig died on the twenty-second day and had a marked necrotic enteritis. *Salmonella choleraesuis* was recovered from the pig fed the three, 25-cc. doses and the contact pig, but not from the other 3. The results show the culture to be pathogenic at this time.

Experiment 2.—In this experiment, 12 pigs raised at the Animal Disease Station, weighing from 60 to 100 lb., were each fed 25 cc. of

TABLE I—Results of Intravenous Injections of 46 Normal Pigs with 24-Hour Broth Cultures of Salmonella Organisms

Strain used	Length of time after isolation of strain	No. pigs injected	Results
S. choleraesuis:			
Strain 4623L	1 to 5 years	12	12 dead within 72 hours
Strains from pigs with acute infection (5 pigs injected with strain 4601)	Within 7 days	14	14 dead within 72 hours
Strain O	1 month	2	1 dead in 4 days; 1 dead in 6 days
Strain 4601	3 months	9	2 dead within 72 hours; 2 dead in 5 days; 3 dead in 19-49 days; 2 recovered
Pooled strains 4601 and P	1 year	2	1 moribund and destroyed in 18 days; 1 recovered
Strain 4601	14 months	4	1 dead within 72 hours, 3 recovered
S. seuftenberg	7 months	1	Recovered in 5 days
S. worthington	7 months	1	Recovered in 7 days
S. derby	7 months	1	Recovered in 3 days

24-hour broth culture of 4623L (5/29/38) on Nov. 26, 1941. All pigs had fever and were off feed on the second day. Three pigs died, 2 on the sixth day and 1 on the ninth day. All 3 had marked necrotic enteritis at necropsy, and *S. choleraesuis* was recovered from the heart blood and spleen of each pig. The other 9 pigs remained off feed until the fifth day when 5 had fair appetites. All had fair to good appetites by the tenth day. On the twenty-third day, all were apparently normal except 1, which was still unthrifty. The unthrifty pig and 3 of the others were destroyed on the twenty-third day. There were some slight necrotic lesions in the colon and cecum of the unthrifty pig but the intestinal tracts of the other 3 were normal. The other 5 pigs were killed on the forty-second day. The intestines were normal.

The daily maintenance ration of 6 of these pigs had been supplemented with 200 mg. of nicotinic acid. No significant difference was observed between the 6 pigs on supplemented ration and the 6 pigs on unsupplemented ration.

Salmonella choleraesuis was not recovered from any of the 9 pigs that were destroyed. The results show that the culture was still pathogenic and do not indicate any change in degree of virulence from that shown in the first experiment.

Experiment 3.—In this experiment, 9 pigs, raised at the Animal Disease Station, were fed an avirulent culture of *S. choleraesuis* 4623. This culture was the same as strain 4623L (5/29/38), except that it had been carried on regular stock mediums instead of being lyophilized. Twenty-four-hour broth culture of this avirulent strain produced no detectable ill effects in mice when injected subcutaneously in amounts of 0.5 cc. The 9 pigs were fed 5 cc. of 24-hour broth culture of this avirulent strain each when 13 days old, 10 cc. each when 25 days old, and 25 cc. each when 38 days old. These feedings produced no detectable ill effects in the pigs. They were weaned at 9 weeks of age. They were always thrifty and weighed from 22 to 29 lb. when 58 days old. Four weeks after weaning, 1 of these pigs was observed to be unthrifty. It was sacrificed and showed a marked necrotic enteritis. *Salmonella choleraesuis* was not recovered.

On Jan. 21, 1942, when the surviving 8 pigs were 122 days old, they were each fed 25 cc. of 24-hour broth culture of strain 4623L (5/29/38). At the same time, each of another group of 8 pigs obtained from the Beltsville, Md., herd of the Bureau's Animal Husbandry Division was also fed 25 cc. of 24-hour broth culture of strain 4623L (5/29/38). These animals weighed from 40 to 60 lb.

On the second day after exposure, all pigs had temperatures ranging from 104.4 to 107.8 F. At that time, pigs in the first group were all off feed, whereas of those in the second group, 3 had good appetites, 3 had slight appetites, and 2 were off feed. On the fifth day after exposure, 3 in the first group were off feed with temperatures of 104.2 to 104.4 F., 3 had good appetites with temperatures of 103.2 to 104.4 F., and 2 had slight or fair appetites with temperatures of 103.6 to 105.4 F. At the same time, 2 in the second group were off feed with temperatures of 104.0 to 105.8 F., 2 had good appetites with temperatures of 103.8 to 105.8 F., and 4 had slight or fair appetites with temperatures of 103.4 to 105.8 F. On the fifteenth day, 4 pigs in the first group were apparently normal and 4 had fair appetites, and 7 in the second group were apparently normal and 1 had a fair appetite. At this time, all 16 pigs had lost an average of about 10 lb. each. Four pigs from each group were sacrificed fifteen days after exposure. Necropsies showed no necrotic lesions in the intestines. *Salmonella choleraesuis* was not recovered. The other 8 pigs were sacrificed on the twenty-ninth day. There were no necrotic lesions in the intestines and *S. choleraesuis* was not recovered from any of the tissues. While the results show that the culture was still pathogenic, failure to produce any deaths might well suggest that it had lost some degree of its virulence.

Experiment 4.—This experiment was set up to test the virulence of different strains of *S. choleraesuis* on different types of pigs, in order that some information on this point might be available preparatory to initiating an experiment on the relationship of *S. choleraesuis* to so-called hog-cholera breaks. Thirty-six pigs were used in the test. Twenty-four were fed broth cultures and 12 were used as contacts. The 24 pigs that were fed cultures consisted of 6 boars averaging about 250 lb. in weight, 6, 4-month-old shoats averaging about 50 lb., 6, 10-week-old pigs averaging about 30 lb., and 6, 7-week-old suckling pigs averaging about 15 lb. The 12 contact pigs averaged about 60 lb. The 6, 4-month-old shoats and the 12 contacts had been treated with hog-cholera antiserum and hog-cholera virus two months previously and were obtained from the Animal Husbandry Division. The other 18 had not been treated and were raised at the Animal Disease Station.

The 24 pigs that were fed culture were divided into three groups, each consisting of 2 animals of the four types. The boars and the 4-month-old shoats were penned together, and the 10-week-old pigs and suckling pigs were penned together. Two contacts were added to each pen four days after the other pigs had been fed culture.

Each of the 2 sucklings in each group was

TABLE 2—Effects of Feeding Broth Cultures of Salmonella Choleraesuis to Swine

Pig No.	Litter designation	Age at time of exposure* (days)	Weight (lb.) At weaning	Weight (lb.) 3 weeks after exposure	Weight (lb.) 5 weeks after exposure	Strain designation	Postexposure data: Temperature of 104 F. or above — First day	Number of days	Maximum	Days sick — Marked	Slight	Days having diarrhea	Appetite — Off feed	Slight	Fair	Date of death	Date recovered from exposure	Mortality (%)
Exposed to *Salmonella choleraesuis*																		
7768	A	75	33	35.0	41.5		3rd	4	106.6	1	0	4	1	1	1	.....	11/13	
7775	D	65	27	...	...		1st	5	106.2	4	1	2	4	2	0	11/11	.....	
7788	B	64	29	...	...	4623L	2nd	6	106.6	5	1	7	4	2	0	11/14	.....	87.5
7793	E	62	40	...	...	(1/21/42)	1st	5	106.6	4	0	3	5	0	0	11/12	.....	
7779	C	64	26	...	...		1st	8	106.6	1	2	4	1	2	2	11/13	.....	
7786	C	64	22	...	...		1st	5	107.0	3	0	3	3	0	0	11/10	.....	
7770	A	75	30	30.5	37.5		1st	14	107.8	2	1	8	2	0	2	.....	11/18	
7776	D	65	25	...	...		1st	5	107.8	4	0	2	3	1	0	11/11	.....	
7790	B	64	32	...	...	5812L	1st	6	107.0	4	1	15	0	5	11	11/24	.....	
7795	E	62	19	...	...	(7/10/42)	2nd	15	107.0	2	3	14	2	5	6	11/21	.....	87.5
7780	C	64	27	...	...		2nd	6	107.4	1	4	5	1	4	0	11/12	.....	
7785	C	64	30	...	...		1st	12	107.0	5	3	10	0	8	3	11/19	.....	
7771	A	75	34	...	...		1st	11	107.4	7	5	11	3	9	1	11/20	.....	
7777	D	65	27	...	...	HOWARD	1st	4	107.0	4	1	3	3	2	0	11/11	.....	
7787	B	64	29	...	...	isolated	1st	11	108.4	4	2	8	3	3	2	11/16	.....	
7794	E	62	32	...	...	(11/1/47)	1st	11	108.4	5	3	9	3	5	2	11/17	.....	100
7781	C	64	19	...	...		1st	5	107.2	1	2	3	1	2	0	11/10	.....	
7784	C	64	25	...	...		1st	5	107.6	2	1	4	1	2	0	11/11	.....	
7774	A	75	30	...	...		1st	7	106.8	6	2	7	2	6	1	11/16	.....	
7778	D	65	27	...	...		1st	8	107.0	3	5	5	3	5	0	11/13	.....	
7792	B	64	30	18.5	...	5812L	2nd	11	106.8	2	2	22	1	4	7	11/29	.....	
7796	E	62	40	...	...	(8/6/46)	3rd	11	107.8	2	4	11	2	4	6	11/20	.....	100
7782	C	64	26	...	...		2nd	3	107.0	2	1	4	2	1	0	11/9	.....	
7783	C	64	21	...	...		1st	5	106.8	4	1	4	5	0	0	11/12	.....	
Litter mates not exposed to *Salmonella choleraesuis*																		
7769	A	75	33	57.5	76.5	...	...	..	...	..	..	..	..	..	..	.....	.....	....
7772	A	75	26	51.75	69.0	...	...	..	...	..	..	..	..	..	..	.....	.....	....
7773	A	75	23	46.0	61.5	...	...	..	...	..	..	..	..	..	..	.....	.....	....
7789	B	65	31	47.75	67.5	...	...	..	...	..	..	..	..	..	..	.....	.....	....
7791	B	65	29	45.0	61.0	...	...	..	...	..	..	..	..	..	..	.....	.....	....

*Date of exposure, Nov. 5, 1947.

drenched with 12.5 cc. of 24-hour broth culture, whereas each of the other 6 in each group was fed 25 cc. of 24-hour broth culture individually on feed. The pigs had not had access to feed during the 36 hours just prior to exposure. They were fed the culture on July 11, 1942. One group was fed strain 5812, which was a broth culture prepared from an agar slant that was used the day before in the preparation of 5812L (7/10/42). The second group was fed strain 2136, which was isolated on June 30, 1942, from a hog-cholera virus produced on June 26, 1942. Agar slants of this culture were used the day previous to exposure in the preparation of strain 2136L (7/10/42). The third group was fed strain "Smith," which had been recently isolated from a pig affected with hog cholera.

All the pigs that were fed culture had fever and were off feed on the second day following exposure. Five of the 6 boars and 4 of the 6, 4-month-old shoats were apparently normal by the sixth day. The sixth boar was off feed for ten days following exposure and had a continuously high temperature until the twelfth day, when it was apparently normal. This boar was 1 of the 2 that had been fed strain 2136. Of the 2, 4-month-old shoats that were not normal by the sixth day, 1, fed strain Smith, died on the sixth day. The other 4-month-old shoat, fed strain 2136, was apparently normal on the twelfth day. Upon necropsy, the 4-month-old shoat that died on the sixth day showed a diffuse necrotic enteritis, involving the mucous membrane of both the colon and cecum. *Salmonella choleraesuis* was recovered from the heart blood and spleen.

Four of the 6, 10-week-old pigs died, 2 having been exposed with the Smith strain, 1 with strain 5812, and 1 with strain 2136. The 2 fed the Smith strain died on the third day, and 1 fed strain 5812 and 1 fed strain 2136 died on the fifth day. Necropsies of these 4 pigs showed acute inflammation of the colon and cecum, with marked necrotic enteritis in the 1 that died on the fifth day and had been fed strain 2136. *Salmonella choleraesuis* was recovered from the 2 that died on the fifth day but not from either of the 2 that died on the third day. The other 2, 10-week-old pigs survived and were apparently normal on the tenth day.

The 2 suckling pigs, fed strain 5812, died, 1 on the sixth day and 1 on the twelfth day. Necropsies showed marked necrotic enteritis. *Salmonella choleraesuis* was recovered from the heart blood of the 1 that died on the sixth day but not from the 1 that died on the twelfth day. The other 4 sucklings were severely affected. They were destroyed on the thirteenth day when 1 of the 2 fed strain 2136 was apparently normal and the other 3 still had only slight appetites. *Salmonella choleraesuis* was not recovered from any of these 4.

Of the contact pigs, the 4 placed with the pigs exposed to strain 5812 had rises in temperatures on one or more days during the post-contact period. Two of the Smith strain contacts had rises in temperatures on the fourth or fifth day of contact, whereas the temperatures of the other 2 remained normal. One of the strain 2136 contacts had a rise in temperature on the third, fourth, and fifth days, but the temperatures of the other 3 remained normal. One of the strain 5812 contact pigs was off feed on the third day of contact. The appetites of the other 11 pigs were not appreciably affected. All were normal after fifteen days of contact, when they were destroyed. There were no lesions in the stomach or intestine.

The results of the experiment indicated that the three strains were pathogenic and there appeared to be no significant difference in their degree of virulence.

Experiment 5.—In this experiment, 20 pigs, raised at the Animal Disease Station, and weighing from 40 to 60 lb., were used. Like the animals used in experiment 1, these pigs also served as controls in an experiment planned to produce a hog-cholera break. On Aug. 5, 1942, 10 each were fed 25 cc. of 24-hour broth culture of strain 5812L (7/10/42), and 10 each were fed 25 cc. of 24-hour broth culture of strain 2136L (7/10/42). Temperatures of these pigs were not taken following exposure. Of the pigs exposed with strain 5812L (7/10/42), all were off feed or had impaired appetites during the first week after exposure. Five of the pigs died, 3 on the fourth day, 1 on the seventh day, and 1 on the twenty-third day. These 5 pigs had marked necrotic enteritis when necropsied and had septicemias of *S. choleraesuis*. At the end of the fourth week after exposure, the other 5 pigs were back on full feed and apparently normal.

Of the pigs exposed with strain 2136L (7/10/42), most were off feed or had impaired appetites following exposure. Seven died on the fourth, sixth, ninth, thirteenth, twenty-second, thirty-second, and forty-seventh days, respectively, following exposure. Five of the 7 pigs had marked necrotic enteritis when necropsied. The 2 dying on the thirty-second and forty-seventh days, respectively, following exposure had enteritis, but there were no necrotic lesions of the colon or cecum. *Salmonella choleraesuis* was recovered from 4 of the 7. The other 3 had recovered by the tenth day.

On Nov. 11, 1942, each of the 8 survivors in this experiment were injected subcutaneously with 2 cc. of hog-cholera virus. All died of cholera. The results of this experiment show that both strains were pathogenic. The 50

per cent mortality (5 of 10 pigs) and severe reactions in the other 5, resulting from exposure with strain 5812L (7/10/42), as compared with no mortality and more moderate reactions obtained in experiment 3 with strain 4623L (5/29/38), seem to indicate that the virulence of strain 4623L had been increased as a result of the six serial passages made by intravenous injections through swine.

Experiment 6.—This experiment was planned primarily to duplicate a hog-cholera break experiment previously completed. Fecal samples from all pigs were collected at intervals before and after exposure and examined for the presence of Salmonella and for anti-*Salmonella choleraesuis* bacteriophage which had been reported by Dale, Meriweather, and Schoening[32] to be present in the feces of some pigs. All pigs were weighed at regular intervals. Chemical analyses of the nicotinic acid content of five samples of the feeds used were made. Two of the samples were low in nicotinic acid but would provide an adequate amount on the basis of the findings of Powick, Ellis, Madsen, and Dale[33] in connection with their work with synthetic diets. Only the pathogenicity of the strains of *S. choleraesuis* used in the experiment is reported here.

Thirty-two, cholera-susceptible pigs were used in the experiment. The pigs were raised at the Animal Disease Station. Sixteen were farrowed by sows that had survived previous exposure to *S. choleraesuis*, and 16 were farrowed by sows previously used in brucellosis tests. The pigs in the first group were 8 to 10 months old and averaged 139 lb. The smallest pig weighed 70 lb. and the largest 215 lb. Those in the second group were 6 to 7 months old and averaged 112 lb. The smallest pig weighed 76 lb. and the largest 141 lb. The animals were divided into four lots with 8 pigs to each lot, 4 pigs being from the older group and 4 from the younger group. Lot 1 was exposed with strain 5812L (7/10/42), lot 2 with 4623L (1/21/42), and lot 3 with a strain that was resistant (R)* to anti-*Salmonella choleraesuis* bacteriophage. Lot 4 served as a control group, none of the pigs being exposed. Exposures were made on March 15, 1944, by feeding each pig 25 cc. of 24-hour broth culture with a stomach tube. Each lot was isolated in a separate pen.

Of the 8 pigs fed strain 5812L (7/10/42), all had fever on the second day following exposure. One had a good appetite and the other 7 had fair appetites. On the third day, 7 were apparently normal, having good appetites and no fever. The eighth pig had a good appetite but a temperature of 104.8 F. The temperature of this pig was 105.0 F. on the following day and then dropped to normal.

Of the 8 pigs fed strain 4623L (1/21/42), 7 had slight fever, but all had good appetites on the second day following exposure. All were apparently normal on the third day following exposure.

None of the 8 pigs exposed with the bacteriophage-resistant strain of 5812L (7/10/42) showed any symptoms following exposure.

No other symptoms developed in the exposed pigs during an observation period of forty-two days. The control group was normal during the entire period. At the end of the observation period, all pigs were released and no necropsies were made.

On the basis of the results obtained in this experiment, it might easily be concluded that the cultures lacked virulence, although there was the possibility that the pigs were highly resistant. The method of administering the broth culture may also have been a factor that contributed to the minimal response following exposure. To obtain additional information on the susceptibility of the pigs, 2 additional pigs were exposed by this same method on April 12, 1944, with 25 cc. of a 24-hour broth culture of *S. choleraesuis* that was isolated on April 4, 1944, from a pig that died of a field outbreak of hog cholera. One pig came from the same source as the older group, and the other came from the same source as the younger group. Neither pig was noticeably affected, except that 1 pig had a temperature of 104.2 F. on the second day after exposure. This result provided additional evidence that the failure to obtain more severe reactions was due to factors other than a lack of virulence of the cultures.

In connection with other experimental work in which lyophilized strains of 4623 and 5812 had been used and on the basis of the results obtained in the above experiments, it was considered desirable to test the virulence of these cultures on a more selective group of pigs. Accordingly, such a test was made in experiment 7.

Experiment 7.—This experiment was planned to determine the pathogenicity of strains 4623L and 5812L for a group of pigs obtained from the same source and of about the same age as pigs used by Powick, Ellis, Madsen, and Dale.[33] This was particularly desirable because a large number of pigs that they used were exposed to the lyophilized strains 4623 and 5812.

The pigs obtained from the Animal Husbandry Division were the progeny of crosses of inbred lines. The sows farrowed in a cen-

*On March 13, 1944, a lyophilized vial of strain 5812L (7/10/42) was seeded in broth. After seven hours' incubation, broth culture was spread over the surface of an agar slant. Immediately afterward, the seeded surface was covered with bacteriophage. After eighteen hours' incubation at 37 C., four or five colonies were visible on the seeded surface of the agar slant. Broth was seeded with bacteria from one of these colonies. After twenty-four hours' incubation, the broth culture was used for exposure of pigs in lot 3.

tral farrowing house, and within a week each sow and her litter were moved to an individual pen in an isolated building. The pigs had access to a graveled runway leading from each pen.

The pigs thrived during the preëxposure period and were weaned when 56 days old. At that time, the average weight of the pigs was slightly less than that of comparable pigs from sows that were moved to areas where they had access to pasture. The sows, moved to the isolated building, were not fed heavily and were rather thin when their pigs were weaned. The weights of the pigs at weaning time are recorded in table 2.

The strains used for exposure of the pigs were 4623L (1/21/42), 5812L (7/10/42), 5812L (8/6/46), and Howard. Strain 5812L (8/6/46) was prepared from strain 5812L (2/16/44), which was prepared from 5812L (7/10/42). The Howard strain was isolated on Nov. 1, 1947, four days before it was used in the test.

Twenty-four pigs were assigned to four groups so that each of five litters was represented equally in each group. In addition, 3 pigs from litter A and 2 pigs from litter B were held as unexposed controls.

Each of the four groups was exposed with a different strain. Feed was withheld from the pigs for thirty-six hours prior to exposure. Each pig was exposed with 25 cc. of 24-hour broth culture mixed with the feed. The four groups had been moved to isolated pens at the Animal Disease Station two days before exposure.

Twenty-two of the 24 exposed pigs died within four to twenty-four days after exposure. Details of the clinical symptoms are recorded in table 2. *Salmonella choleraesuis* was recovered from the tissues of 20 of the 22 pigs that died.

Necropsies of the 22 pigs that died showed marked necrotic enteritis in addition to the usual lesions suggestive of septicemia. The necrotic lesions were diffuse and affected the entire mucous surfaces of the colon and cecum. The necrosis was superficial in those animals that died early, but the deeper tissues became involved proportionately to the length of survival. At the time of necropsy, the spleens were removed and stored in a deep freeze refrigerator. A bacteria-free filtrate of a saline suspension prepared from portions of spleens from pigs 7779, 7785, 7771, and 7796 produced no reactions when injected subcutaneously into 2 cholera-susceptible pigs and 1 cholera-immune pig. After an observation period of thirty days, 1 of the cholera-susceptible pigs was injected with hog-cholera virus and proved to be susceptible.

While 1 pig exposed with strain 4623L (1/21/42) and the litter mate exposed with strain 5812L (7/10/42) survived, they were rather severely affected. The differences in the results obtained with the four different strains were not sufficient to be considered significant.

DISCUSSION

Some investigators are of the opinion that the enteritis caused by feeding cultures of *S. choleraesuis* to swine is a laboratory creation rather than a basis for determining the etiology of types of enteritis that occur in the field; and this opinion is not without foundation. However, it has been by this means that much of the present day knowledge on *S. choleraesuis* infection has been obtained.

In using the feeding of broth cultures of *S. choleraesuis* to swine in experimental work, investigators have had difficulty in obtaining the same results when attempting to repeat an experiment. Failure to obtain the same results has often been attributed to the possible variation in the relative virulence of the cultures used for exposure. Attempts have been made to overcome this difficulty by increasing the virulence of the strain used for exposure by animal passage and by increasing the amount of culture fed to the experimental animals. In spite of these procedures, variation in virulence of the culture used for exposure has not been eliminated as a possible cause of such variable results. Consequently, it has been difficult or impossible to determine the relative resistance of exposed pigs.

It was recognized early that if the virulence of a strain of *S. choleraesuis* could be maintained by lyophilization, it might provide a method for better evaluating the results obtained in various experiments. While the results in some of the early experiments described here indicated that the virulence of strains of *S. choleraesuis* could be maintained by lyophilization, failure of the cultures used in experiment 6 to produce any appreciable ill effects in the exposed animals might well have justified the conclusion that they did not possess any appreciable virulence. However, results in some concurrent unpublished experiments, in which pigs that had been maintained on purified diets were exposed with some of the same cultures, did not warrant such a conclusion. The high mortality and severe reactions obtained in the 6 pigs exposed with strain 4623L (1/21/42) in experiment 7, as compared with the slight effect produced in the 8 pigs fed the same strain in experiment 6, make it appear definitely that the differences in the results were not due to variation in the virulence of the culture. They may have been due, in part, to the differ-

ence in the methods of administering the broth culture. In experiment 6, administration of the culture was made with a stomach tube, whereas in experiment 7, the culture was mixed with the feed. Rasmussen, Stafseth, Freeman, and Miller[22] reported that they obtained more severe reactions when the culture was administered in the feed than when given by drenching. If the severity of reactions may be influenced by the different methods of exposure, the results obtained in experiment 7 indicate that the reactions are most marked when the culture is given in the feed. Slavin[14] reported that infection of swine with *S. choleraesuis* was obtained by spraying lightly the grass of a new pen with broth culture just before the newly weaned pigs were placed in it. He also found that pigs could be infected by contact during the early stages of an outbreak and by placing them on ground vacated a few hours previously by recovered pigs. It would seem that further investigations are needed to determine definitely the extent of variation in results that may be obtained by the different methods of exposure.

Experiments reported by Gwatkin and Moynihan,[28,29] with strain 5812, suggested that this culture possessed more virulence when used soon after it was removed from the lyophilized state than later. The authors do not report whether they continued to carry the culture as a lyophilized one or on a culture medium.

No evidence was obtained to indicate that any specific factor was responsible for the high susceptibility of the pigs used in experiment 7.

SUMMARY

Reports are recorded on the pathogenicity for swine of lyophilized cultures of two strains of *Salmonella choleraesuis*. One of the strains (4623) was isolated on March 29, 1937, and lyophilized on April 6, 1937. The second strain (5812) was isolated July 9, 1942, and lyophilized July 10, 1942.

Of 43 normal pigs, 26, injected intravenously with 24-hour broth cultures of *S. choleraesuis* that had been maintained by lyophilization or used within seven days from the time they were obtained from the heart blood or spleens of swine, died within seventy-two hours. Of the remaining 17 pigs injected with 24-hour broth cultures of *S. choleraesuis* that had been carried on culture mediums from one to fourteen months, 3 died within seventy-two hours, 8 died or were moribund within four to forty-nine days, and 6 recovered.

Three of 18 pigs that may have had some immunity to *S. choleraesuis* infection and that were injected intravenously with 24-hour broth cultures of *S. choleraesuis* carried on culture mediums from one week to five months died within seventy-two hours. Of the remaining 15 pigs, 9 died or were moribund within four to thirty-three days, 1 had a severe reaction but recovered by the eleventh day, and 5 had slight reactions but recovered in from five to seventeen days.

In seven experiments carried out between October, 1940, and November, 1947, 124 pigs were fed 24-hour broth cultures of *S. choleraesuis*, usually in 25-cc. amounts. Results varied from no appreciable effects in one experiment to a mortality of 22 out of 24 in another experiment. The strains of *S. choleraesuis* used had either been preserved by the process of lyophilization for periods of three to ten years or isolated from the heart blood or spleens of pigs that had died with septicemias of *S. choleraesuis* within a week prior to their use. Broth cultures of one strain that had been lyophilized for seven years produced no appreciable ill effects when fed to 8 pigs in one experiment, but almost four years later, the same lyophilized strain killed 5 out of 6 pigs, and produced a severe reaction in the sixth pig.

CONCLUSION

The results of intravenous injections and feeding of broth cultures of *Salmonella choleraesuis* preserved by the process of lyophilization indicate that the pathogenicity of this microörganism for swine can be maintained by this method as an invariable factor for a period of at least ten years.

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Sludged Blood in Three Young Pigs Experimentally Infected with Hog Cholera

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THE PURPOSE of this paper is to report a preliminary, "pilot" set of microscopic observations of the circulating blood of healthy, normal pigs (*Sus scrofa*), and of the same pigs after they had been infected with hog-cholera virus (*Tortor suis*).

This is a direct extension of previous studies made to determine the changes in the mechanical consistency of the blood, rates of blood flow, pathologic permeability of vessel walls, and vessel contraction patterns in human beings suffering from a variety of pathologic conditions and diseases.[4]

MATERIALS AND METHODS

Three small, healthy, normal pigs, each estimated to weigh about 25 lb., and known to be cholera-susceptible, were selected for the study. This selection is important because no study of pathologic physiology can show its results clearly unless it is known with certainty that the animals were healthy to begin with.[2] The animals were not anesthetized but simply held on the table by hand, and the circulating blood studied in the small vessels of the bulbar conjunctiva at magnifications of 48 x and 64 x. The area was obliquely illuminated with a Shahan ophthalmoscopic lamp and the observations made with a bi-objective, stereoscopic, Leitz microscope. This method permits microscopic study of a statistically valid sample of all of the subject's circulating arterial blood.[4]

This procedure proved to be rather easy to do, but also demonstrated that if any long series of studies of this kind are to be carried out, special, quite simple holding apparatus, probably consisting of flat straps and firm supporting pads, should be prepared to restrain the animals and keep them uninjured while they are observed.

Each of the 3 pigs had essentially normal circulating blood and bulbar conjunctival vessel walls, as judged by the microscopic criteria previously established.

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OBSERVATIONS

1)—The red cells were unagglutinated and tended to repel each other slightly. Each was free from the others. Rouleaux were not present. This could easily be determined by the laminar flow patterns and the behavior of blood in vessels where it was temporarily stationary. In these blood vessels, red cells showed no tendency to come together and agglutinate.

2)—Leucocytes were not sticking to healthy, uninjured endothelium.

3)—The flow of the unagglutinated blood was laminar or streamlined.

4)—Small vessels were not leaking fluid fast enough to cause microscopically detectable hemoconcentration or edema.

5)—The flow in most vessels of 60 to 120 μ internal diameter was so rapid that individual red cells could not be seen. This is a crude but accurate and useful criterion for adequate rates of blood flow through most open tissue capillaries. If the rates of flow of oxygen-saturated arterial blood are fast enough, so that individual, unagglutinated blood cells can not be seen in vessels this size in animals and human beings, high magnifications have shown that no visible hemoconcentration is taking place in true capillaries, *i.e.*, almost no blood fluid is being lost through the walls of tissue capillaries.

6)—The shapes and dimensions of the vessels of the visible part of the peripheral vascular beds constitute a perpetual bottleneck in the circulatory system. All the vessels which carry blood toward tissues are long, narrow, slowly tapering, truncated cones. When arterioles branch, each branch has a narrower lumen than that of the parent stem, etc. (For more extensive details, *see* Knisely, Bloch, Eliot, and Warner, 1947.)[4]

These pigs also showed a special type of pigmentation of the bulbar conjunctival tissues, which proved most helpful for these observations. Because it may be quite useful in the future, it is herein described. Over considerable areas of the bulbar conjunctiva, there was a rather uniform scattering of small black spots. When seen through the microscope, these spots gave the tissue the approximate appearance of a finely stippled drawing. (The spots were not identified, but one might hazard a temporary guess that they were melanophores or small clusters of melanophores.) The

significant feature for this type of observation was their relationship to the small blood vessels of the bulbar conjunctiva.

The sharply stippled effect was confined to areas *between* blood vessels. Further, on each side of each vessel there was a narrow, easily visible band of tissue in which no small black spots were present. These non-pigmented stripes could not be mistaken for the peripheral plasma layers of the blood flowing within the vessels, for the peripheral plasma layers were also visible. Thus, seen at low magnifications, the bright red stripe of the axial stream of the flowing blood had a bright, wide band of sharply contrasting white tissue on each side of it, and at the outer border of each white band, the darkly stippled tissue began.

The significant feature for this type of observation is that the sharply white bands continually mark the anatomic position of each small blood vessel. When a vessel contracts so tightly that it has no lumen throughout its length, the pair of parallel, unpigmented bands of tissue on each side of it are brought together, making one wide, white band, which stands out as clearly as a long concrete highway seen from an airplane. This unmistakably marks the anatomic position of the vessel, which has itself entirely disappeared from view. Thus, when studying these areas of the bulbar conjunctiva of pigs, the vessels conducting blood or containing blood are bright red, and all the exact positions of all tightly contracted vessels are represented by the wide, shining white streaks. As will be shown in the section describing the observations made when the pigs were in an advanced stage of cholera, this is most useful anatomic knowledge.

Duroc-Jersey pigs were used in this experiment. It seems worthwhile to observe other breeds of pigs to see if they have this same pigment distribution in the bulbar conjunctiva.

These microscopic observations made in healthy, normal, cholera-susceptible pigs were carried out on July 22, 1948.

On July 23, each pig was inoculated subcutaneously, in the axillary space, with 2.0 cc. of commercially prepared (serial No. 813) hog-cholera virus (*Tortor suis*). The animals became ill and their symptoms, consisting of elevated temperature, anorexia, leucopenia, diarrhea, and apathy followed rather standard patterns for hog cholera. Their daily temperature readings are recorded in figure 1. Their circulating blood and bulbar conjunctival vessels were observed one week after the inoculation, on July 30 and on July 31. The essential findings were as follows:

All, or almost all, the red cells were ag-

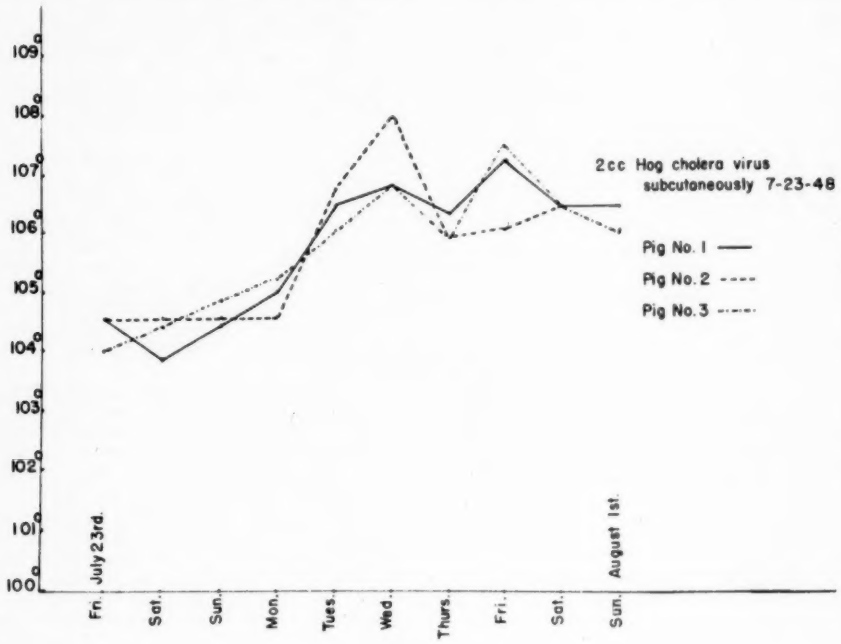

Figure 1

glutinated. The basic masses thus formed were, for the most part, quite small and rather soft. The basic masses of a sludge have been defined as those which do not break up into smaller ones as they pass through the terminal arterioles and capillaries.[4] The sludge in each of these cholera-infected pigs was fairly homogeneous, consisting of small basic masses, all approximately the same size.

The basic masses were so small and soft that they did not bulge the terminal arterioles as they passed through. Hence, the basic masses of this sludge did not seem to present much resistance to their own passage down the visible, cone-shaped arterioles. Thus, *at this rather late stage of hog cholera in these 3 pigs, the resistance of this particular type of sludge* to its own passage through the visible vessels did not seem to be of major significance.

In some few areas in small venules, the small basic masses showed what we have been calling "charge agglutination." That is, in some cases when the flow in a venule was quite slow, the basic masses came together forming larger, cohering aggregates. These were, for the most part, quite soft and broke up into the basic masses again easily as the blood in the small venule was carried down into a larger venule having a faster flow.

The most significant, visible finding was that, over large areas of each pig's bulbar conjunctiva, most vessels had contracted and become so tightly shut that they had no lumen and thus, neither conducted nor contained blood. This, as the previously described anatomy makes clear, was most conspicuous because of the great numbers of shining white stripes crossing the darkly stippled bulbar conjunctiva.

Human Blood.—Some observations previously made upon unanesthetized human blood donors may provide a clue leading toward an understanding of why so many of these vessels were shut off tightly for long periods. Thirty-four persons have been studied by focusing a microscope on the obliquely illuminated bulbar conjunctiva before, during, and after they had donated the usual amounts of blood to a hospital blood bank. Each was unanesthetized and was traumatized only to the extent of being stuck with the large, hollow needle through which the blood was drawn. When between 300 and 400 cc. of blood had been drawn from each subject, one set of vessels and then another of the conjunctiva began to contract and close tightly, and many such sets did not reopen for some time.[4] This is direct evidence that in response to a significant decrease in circulating blood volume, parts of the vascular system shut off tightly.

Other observations made by the fused, quartz rod method inside anesthetized vertebrates, including Rhesus monkeys, leads one to suspect strongly that when the vessels of one area, which consists largely of connective tissues, shut off tightly, all or most areas of the body, which also consist largely of similar connective tissues, also have their vessels clamp tightly shut.

Thus, the observation that many vessels were contracted and closed tightly in the bulbar conjunctiva of these cholera-infected pigs logically leads to the strong suspicions that: (1) The animals had a pathologically low circulating blood volume; and (2) in consequence, the small vessels in many connective tissues, and perhaps other types of tissue, were so tightly shut that the cells of these tissues remained underoxygenated and undernourished for long periods. More evidence for this is presented by Knisely, Stratman-Thomas, Eliot, and Bloch (1945)[2] and Knisely, Bloch, and Warner (1948, especially p. 56).[5]

In addition to the previously mentioned, apparently significant, prolonged vessel spasms, some few small venules showed the pathologic sacculated dilatations described by E. R. and E. L. Clark.[1] (This paper is a classic on the pathologic responses of the walls of small vessels to injury and should be studied by all who are interested in circulatory pathology.)

Further, in some areas of the bulbar conjunctiva of these sick pigs, some few small venules were plugged by the previously mentioned, soft charge aggregates.

Progressive hemoconcentration of the passing blood could be observed in some of the postcapillary venules and small venules, which is evidence that their vessel walls were leaking fluid. In agreement with this, most of the bulbar conjunctiva of each animal showed easily visible degrees of local tissue edema. The evidence for the presence of edema is that the tissues were slightly swollen, glassy, and it was difficult to focus the microscope on deeply lying structures. (This is the condition called "chemosis" when seen in human patients.)

It seems quite possible that the basic masses, consisting of agglutinated red cells, were being phagocytized and destroyed in the liver and spleen of these cholera-infected pigs. In a long series of microscopic observations in the living, quartz rod, transilluminated livers of frogs and Rhesus monkeys, it has been observed that normal, naked, unagglutinated red cells are never ingested by the sinusoid-lining, phagocytic von Küpffer cells.[2, 5]

In contrast, during stage 2 of *knowlesi* malaria in monkeys, malaria-parasitized red cells become coated with a sticky precipitate, agglutinated into small masses, and

these sticky, small masses of parasitized red cells have been directly observed to be rapidly ingested by the monkey's hepatic sinusoid-lining phagocytes.[2]

Further, the fact that the circulating red cells stick together in small masses in these pigs may be taken as evidence that the surfaces of these cells have been altered from their healthy, normal condition; it may, perhaps, be taken as evidence that these cells are coated with thin films of sticky precipitates[5] (p. 61). The nature of the surface of the coating around a small mass, rather than the nature of the materials contained within the coating, determines whether the coating, and inescapably its contents, are selectively phagocytized from the circulating blood[5] (p. 81).

It is known that samples of blood drawn from pigs with cholera frequently have low red cell counts, *i.e.*, various degrees of anemia. In Rhesus monkeys infected with *knowlesi* malaria, the rapid phagocytosis of coated, agglutinated masses of red cells is a major factor causing anemia and low circulating blood volume.[2] Hence, at present, it seems quite possible that in pigs with hog cholera, rapid phagocytosis of agglutinated, red blood cells may be a major factor in initiating and maintaining: (1) low red cell counts, and (2) severely pathologic degrees of low circulating blood volume.[4]

All 3 pigs were killed for postmorten examination, particularly to see if they had infections other than, or in addition to, hog cholera. Pig 2 was killed and autopsied on July 31, and pigs 1 and 3 on August 1. Briefly, the protocols on them are as follows:

Pig 1.—Immediately before sacrifice, the white blood cell count was 8,600; red blood cell count, 5,530,000; hemoglobin, 9.5 Gm. per 100 cc; the sedimentation rate was approximately 13 mm., or 35 per cent of the column in thirty minutes. This animal was killed by exsanguination.

Pig 2.—The white blood cell count was 4,500; red blood cell count, 5,900,000; hemoglobin, 11 Gm. per 100 cc.; sedimentation rate, 11 mm., or 30 per cent of the column in thirty minutes. This animal was destroyed by electrocution.

Pig 3.—The white blood cell count was 7,200; red blood cell count, 5,430,000; hemoglobin, 10 Gm. per 100 cc.; sedimentation rate, approximately 10 mm., or 25 per cent of the column in thirty minutes. This animal was killed with 10 cc. of nembutal.

All 3 animals were moribund at the time of sacrifice. In general, the lesions noted grossly at autopsy were peripheral hemorrhages of the skeletal and visceral lymph glands, petechial hemorrhages of the kidney cortex and medulla and of the serous surfaces and mucous membranes of the urinary bladder and larynx. All 3 animals showed petechial hemorrhages in the brain, and 1 animal showed a large submeningeal ecchymosis on the posterior left cerebral hemisphere.

Bacteriologic examination of the liver, spleen, and kidney of each pig on blood agar and MacConkey's agar failed to reveal evidence of known pathogenic organisms.

On the basis of the observed lesions, together with the lowered total leucocyte count and absence of known pathogenic bacteria in the cultures, it was concluded that these pigs were affected with uncomplicated hog cholera.

This brief preliminary study obviously raises many more questions than it answers.

The most important possible conclusion is that a set of studies should now be initiated on a statistically significant series of pigs, aimed at finding valid answers to some of the possibilities herein described.

SUMMARY

The circulating blood and blood vessels of the bulbar conjunctiva of live pigs were studied with a high power dissecting microscope before and after inoculation with hog-cholera virus (*Tortor suis*). Prior to inoculation, the blood vessel walls appeared normal.

Seven days after inoculation with hog-cholera virus, all, or almost all, the red cells were agglutinated into small, soft masses which did not bulge the terminal arterioles. In some cases, these basic masses came together, forming larger, cohering aggregates, which broke up again quite easily. Over large parts of the conjunctiva of each pig, most vessels had contracted, obliterating the lumen. A special type of pigmentation of the bulbar conjunctiva, which is described in detail, made this contraction clearly visible.

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Pathogenesis of Bovine Mastitis
I. The Relation of Age to Streptococcic Infection

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STREPTOCOCCIC infection of the bovine udder has been observed more often in older cows than younger ones. Seelemann[1] found an average incidence of 8.6, 30.1, 42.0, 44.1, 51.7, 51.5, and 56.3 per cent infection in successive lactation periods. He based his results on 6,834 cows examined over a two-year period. The reasons for the higher incidence in older cows are important in relation to prophylaxis and control of streptococcic mastitis.

In several recent publications, Murphy[2,3,4,5] suggested that the increasing incidence of infection with age is due to the aging process within the cow and not to the longer exposure to infection. He presented data from two herds studied over a period of seven years. The degree of exposure to streptococci has previously been considered of major importance in streptococcic infection of the udder. Measures to reduce the degree of exposure, such as segregation and sanitation, have been effective in control as shown by Minett,[6] Little and Plastridge,[7] and many other workers.

Further information on the relation of age to streptococcic infection seemed desirable. Therefore, the results of herd studies during the past several years were examined to determine if they conformed to previous reports and if conclusions could be drawn from them concerning the causes and importance of the increasing incidence with age.

METHODS

A bacteriologic examination was made on the quarter samples from each cow in dairy herds as a part of several experimental mastitis control programs. The methods used were the Hotis test, microscopic examination of incubated samples, and streak-plating of the incubated sample on either blood agar or azide-blood agar. Further details of the testing procedures have been reported by Spencer *et al.*[8] Since the incidence of streptococcic infections other than *Streptococcus agalactiae* was less than 10 per cent, all streptococcic infections were considered together. Tests were made at intervals, but only one test result was used as a basis for this report.

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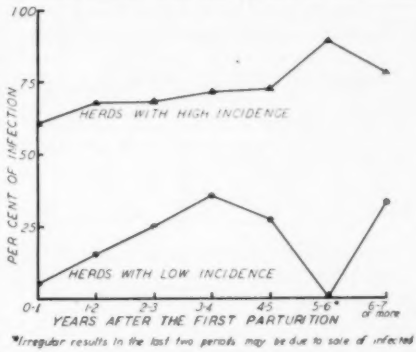
Information was utilized from 12 Holstein-Friesian herds in which chemotherapy had not been practiced, and in which the conditions of herd management had been fairly constant for several years. The ages of the cows were obtained from the herdsman. In some herds, the age was determined accurately from registration or parturition records. In other herds, the age was known to the nearest year. The date of the first parturition was also recorded, when available, and was used as a basis for determining the time from the first parturition to the bacteriologic test. When the date of first parturition was unknown, it was assumed to have occurred at the age of 2 years.

The conditions of management were observed over a period of one to seven years in each herd. The degree of segregation, milking order, and methods, sanitation, and feeding practices were all considered as factors of management that might possibly affect the incidence of streptococcic infection.

RESULTS

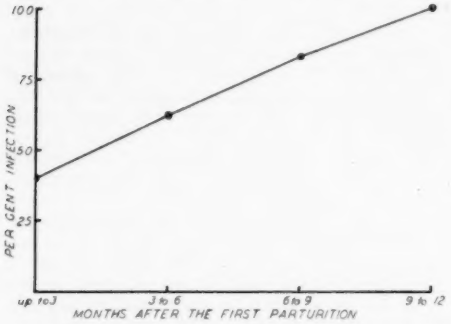
Table 1 shows the number of infected and noninfected cows in the several age groups of each herd. The incidence of herd infection with streptococci varied from 10.7 to 86.6 per cent. Infection in the majority of the herds varied from 60 to 80 per cent. The incidence was low in two herds and high in the other ten.



Graph 1—The incidence of infection compared to the time after the first parturition. Ten herds with a high incidence and two herds with a low incidence.

Table 2 combines the data from herds 1 and 2, showing the percentage of infection

for each successive year of lactation. A regular, linear increase in incidence occurred during the first four years of lactation. Irregular results were observed for later periods, possibly because of the small numbers of animals in those groups or sale of infected animals. There were only 4 infected animals out of 17 in the fifth or later years of lactation. This is an indication that the regular, yearly increase of incidence probably was slowed or halted after the fourth year in these two herds.



Graph 2—The incidence of infection compared to the time after the first parturition. Highly infected herd 12.

Table 3 combines the results from the ten herds with a high incidence of streptococcic infection. In these herds, the increase in incidence was not more rapid than from the first to fourth years in mildly affected herds. The incidence in cows examined during their first year of lactation in the badly affected herds was 60.6 per cent. This high incidence during the first year suggests a very rapid rate of infection during the first few months of lactation. A comparison of the incidence of streptococcic infection in the two types of herds is portrayed in graph 1.

The rate of increase in incidence of infection during the first year of lactation in herd 12 is presented in graph 2. Since the number of animals for each monthly period was small, cows were combined to include animals from a three-month period. The result in this herd demonstrates a regular, progressive increase in incidence of streptococcic infection at a rate of about 20 per cent for each successive three-month period. The relationship between duration of lactation and incidence is a linear one, similar to that for herds 1 and 2 as shown in graph 1. In herd 12, the incidence approached 100 per cent in twelve months. In this herd, there were 47 cows which had lactated more than nine months and 45 of them were found to be infected. The 2 older non-infected cows had first freshened twenty-six to twenty-eight months prior to the test.

The management varied from herd to herd and appeared to be related to the incidence of infection. Herds 1 and 2 were managed carefully, and the cows were milked in this order: heifers first, clean cows next, and infected cows last. These herds had been subjected to regular bacteriologic tests for four years to determine the presence of streptococcic infections. The herdsmen practiced good sanitation, disinfection, and excellent milking methods.

TABLE 1—The Occurrence of Streptococcic Infection Among Cows of Different Ages in 12 Herds

Herd	Infection with streptococci	Years since the first parturition							Total cows	Infection (%)
		0-1	1-2	2-3	3-4	4-5	5-6	6 or more		
1	+	0	1	0	1	0	0	1	28	10.7
	−	9	4	5	0	2	3	2		
2	+	1	3	7	6	3	0	0	82	24.4
	−	9	18	16	13	6	0	0		
3	+	0	1	2	1	0	0	3	16	43.7
	−	0	3	1	4	0	0	1		
4	+	0	0	0	4	1	2	3	16	62.5
	−	0	0	0	3	2	0	1		
5	+	0	0	0	4	7	1	0	19	63.2
	−	0	0	1	3	3	0	0		
6	+	5	4	3	8	6	3	7	56	64.3
	−	2	4	5	2	3	1	3		
7	+	1	4	3	6	2	2	2	29	69.0
	−	3	2	0	2	1	0	1		
8	+	1	5	7	7	2	5	6	47	70.2
	−	1	4	4	2	1	1	1		
9	+	0	0	1	3	2	0	4	13	76.9
	−	0	1	0	0	2	0	0		
10	+	0	2	1	2	2	0	0	9	77.8
	−	0	1	0	1	0	0	0		
11	+	0	3	3	0	5	1	0	15	80.0
	−	0	2	0	0	0	0	1		
12	+	13	17	8	8	5	3	4	67	86.6
	−	7	0	2	0	0	0	0		

Herd 6 was moderately well managed, but was hand-milked with no regard for disinfection of the hands between cows. The rest of the herds were poorly managed, with little regard for milking order or sanitation.

The size of the herd was another factor which appeared to be important in the incidence of streptococcic infection. Herds 6 and 12 were large institutional herds with better management than small herds 3, 4, or 5, but the incidence of infection was higher. Herd 2, which was large, was more carefully managed than herd 1, but the incidence of infection was 24.4 and 10.7 per cent, respectively, in the two herds.

DISCUSSION

The results presented in this paper are similar to those of Seelemann and Murphy. Seelemann combined his data from a large number of herds, which presumably had variable conditions and different frequencies of infection. His results seem to indicate that the increase in incidence of infection was greatest during the first year of lactation and declined progressively thereafter. Murphy based his data on two similar herds in the same institution in which he observed a regular rate of infection under uniform conditions over seven years. He found a regular, linear relationship between the age of the animal and the incidence of streptococcic infection. The data reported here were from 12 herds under diverse conditions of management, and in these herds, the incidence of infection also increased regularly with the passage of time following the first parturition. In this work, the rate of increase of infection varied greatly in different herds and appeared to be correlated with factors of herd size and herd management that would influence the degree of exposure.

Murphy rejects the possibility that the degree of exposure to streptococcic infection might be responsible for the increasing incidence in older cows. He thinks that if the degree of exposure were the important factor, the incidence should increase more rapidly with each successive year of advancing age. Apparently, he is considering a herd in which all the cows are the same age. Theoretically, the incidence of infection would increase more rapidly in such a group of cows with advancing age. A herd of that nature was described by Hastings *et al.*[9] and was examined repeatedly during the third lactation by one of the authors (G. R. S.). In that herd, no streptococcic infection was found during the first two lactations, but when the infection was introduced into the herd during the third lactation, it spread to 13 of 24 cows within eight months.

In the herds which Murphy studied and in most ordinary herds, the cows were of various ages. Noninfected heifers were brought into the herd to replace older animals. Thus, in the usual herd, the incidence of infection is kept relatively constant. In the average herd, streptococcic infection is not epizoötic but rather enzoötic, and the factors tending to increase the incidence of infection are offset by factors tending to lower the incidence.

It is the opinion of the authors that the age-incidence relationship is consistent with a uniform degree of susceptibility during the first years of lactation. In herds in which a considerable amount of culling for mastitis was practiced, as in herds 1 and 2, the cows remaining after four years of lactation were likely to be more resistant to streptococcic infection than the average, and the incidence of new infection in these older animals was lower than that in the younger animals. Seelemann's data on 6,834 animals also showed this trend toward a lower incidence of new infection in older animals. Thus, the observed relationship between age and incidence is a gradually decreasing number of new infections with advancing age as in graph 1. Such a relationship would be expected if the average susceptibility of uninfected older animals in the herd was less than that of the younger animals.

TABLE 2—The Incidence of Streptococcic Infection Among Cows of Different Ages in Herds 1 and 2

	Years since first parturition							
	0-1	1-2	2-3	3-4	4-5	5-6	6 or more	Total
No. of cows	19	26	28	20	11	3	3	110
No. infected	1	4	7	7	3	0	1	23
Percentage infection	5.26	15.39	25	35	27.27	0	33.33	20.91

TABLE 3—The Incidence of Streptococcic Infection Among Cows of Different Ages in Herds 3 to 12, Inclusive

	Years since first parturition							
	0-1	1-2	2-3	3-4	4-5	5-6	6 or more	Total
No. of cows	33	53	41	60	44	19	37	287
No. infected	20	36	28	43	32	17	29	205
Percentage infection	60.61	67.92	68.29	71.67	72.73	89.47	78.38	71.43

If the susceptibility of cattle increased with age, as Murphy suggests, the expected relationship of age to incidence theoretically would be little or no new infection during the first few years of lactation and a rapidly increasing incidence of new infection in the older animals. Such a relationship has not been reported with any significant number of animals.

The degree of exposure of any cow to mastitis streptococci would probably depend on several factors, such as the following:

1) Duration of exposure (time spent in the milking herd).

2) Intensity of exposure (number of organisms) determined by:

(a) Incidence of infection in the herd.

(b) Number of organisms shed in the herd milk.

(c) Sanitary measures.

(d) Size of herd.

Of all these variable factors, the first one is most easily measured. The other factors have been assumed to be relatively constant in the herds described here.

That the degree of exposure would appear to be the most important factor in the incidence of streptococcic infection is supported by several facts and observations. The incidence of infection varies with different herds and appears to be lower in herds where management practices tend to reduce the intensity of exposure, and higher in herds where the opposite is true. The incidence of infection appears to be correlated closely with the duration of exposure. The incidence of infection increases regularly with advancing age for several months or years but then tends to remain constant.

Many older cows are infected in only part of the quarters. These animals are susceptible to infection in part of their quarters, and any hormonal, innate physiologic, or aging factors should be the same for all four quarters of these cows. The absence of infection in some quarters of older animals is further evidence that infection is not due primarily to an aging process within the cow, but rather to external factors such as the degree of exposure.

Hormonal factors have been suggested[10] as the cause of the increased incidence with age. There is little evidence supporting such a theory in bovine streptococcic infection. Injection of estrogens was shown by Folley *et al.*[11] to alter the composition of cow's milk, but no record of mastitis was presented. The estrus cycle has not been observed to have an effect on mastitis. In chronic cystic mastitis in the human being, hormonal unbalance is considered possibly important because no infection is present, and the disease occurs during the period of life when estrogenic hormone production is known to be disturbed. This condition in the human being, as described by Moore,[12] does not resemble chronic streptococcic mastitis of the bovine species. Variations in the virulence of streptococci and the resistance of cows are other factors that may affect the incidence of infection and the degree of inflammation. The results of studies on hypersensitivity as it affects the resistance of cows will be the subject of a later report.

SUMMARY AND CONCLUSIONS

In 12 dairy herds, the incidence of streptococcic udder infection increased regularly with advancing age but at different rates in the various herds. The rate of increase of infected animals was correlated with herd management procedures, which affected the degree of exposure. These findings emphasize the importance of the following control measures for streptococcic mastitis:

1) Milking younger animals first.

2) Sanitation, disinfection, and segregation to reduce exposure to infection.

3) Replacement of infected animals with healthy heifers.

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Studies on Calves Experimentally Infected with *Cooperia Punctata* (v. Linstow, 1907) Ransom, 1907

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COOPERIA PUNCTATA (v. Linstow, 1907) Ransom, 1907, is a trichostrongylid parasite occurring in cattle and occasionally in sheep. This nematode is cosmopolitan in distribution.

LITERATURE

Dikmans[1] stated that in the United States *C. punctata* has a much wider geographical distribution than either of two closely related species, *Cooperia pectinata* or *Cooperia oncophora*, having been collected from cattle in the southern and northern states. Porter[2] related that *C. punctata* was found in 91 per cent of 84 cattle examined from scattered points throughout the southeastern area of the United States. In Australia, Roberts[3] placed *C. punctata* sixth in incidence, stating that it was found in 72 per cent of the animals examined. In Puerto Rico, according to Andrews,[4] it ranks third with an incidence of 55 per cent.

The parasite is found in the digestive tract, principally in the upper part of the small intestine. Ransom[5] stated that in addition to their occurring in the lumen of the digestive tract, the adult worms were found to occur in the intestinal mucosa of the upper part of the small intestine. Hung[6] stated that the parasite was capable of producing a necrotic intestinal inflammation and gave illustrations showing the worms within the wall of the intestine. Dikmans (*loc. cit.*) described lesions in the intestines of calves caused by *C. punctata*, stating that some parasites were seen penetrating the mucosa to varying depths, and several encapsulated and partly degenerated parasites were observed in the deeper structures of the submucosa.

Other closely related species have proved of economic importance in cattle and sheep. Baker[7] reported some cases of parasitic gastroenteritis in calves in New York and Vermont, with *Ostertagia ostertagi* and *C. oncophora* being identified as responsible for the condition. Hamoir[8] described an epizoötic enteritis of cattle in Belgium caused by parasitic nematodes, principally *C. oncophora*. Fourie,[9] reporting on an outbreak of verminosis in 18-month-old cattle in South Africa, stated that there was a severe infection with *C. pectinata* in the small intestine, which showed catarrhal enteritis. A "fair number" of hookworms were present and other parasitic species were present in the abomasum and colon. Andrews[10] described nodules in the intestine of sheep caused by still another species, *Cooperia curticei*. However, he stated that in no instance were the larvae observed to penetrate the mucosa, but only came in contact with the epithelium of the crypts.

In view of the wide distribution and high incidence of *C. punctata* and of the importance of closely related species, it seemed desirable that further observations be made on its life history, pathogenicity, and pathology.

MATERIALS AND METHODS

The calves used in the experiments were obtained as day-old calves from dairies in the vicinity of Auburn, Ala., and placed in previously cleaned, concrete-floored barns. These barns were divided into individual stalls, and the fecal material was removed and floors washed daily. The animals were fed milk twice daily from nipple buckets until about 4 months of age. Alfalfa hay and grain were placed before them when they were about a week old and continued as their regular diet after weaning.

Larvae for the initial infection were obtained by culturing eggs from freshly collected *C. punctata* females. These females were removed from intestinal contents and washed to remove debris. They were then chopped up to free eggs from the uteri and mixed with sphagnum moss and boiled sheep feces. Third stage larvae isolated from three such cultures were employed to infect calf 117. Once eggs were being eliminated by calf 117 and successively infected calves, larvae were obtained by culturing large quantities of fecal material in sphagnum moss in the manner described by Cauthen.[11] The time required for the prepara-

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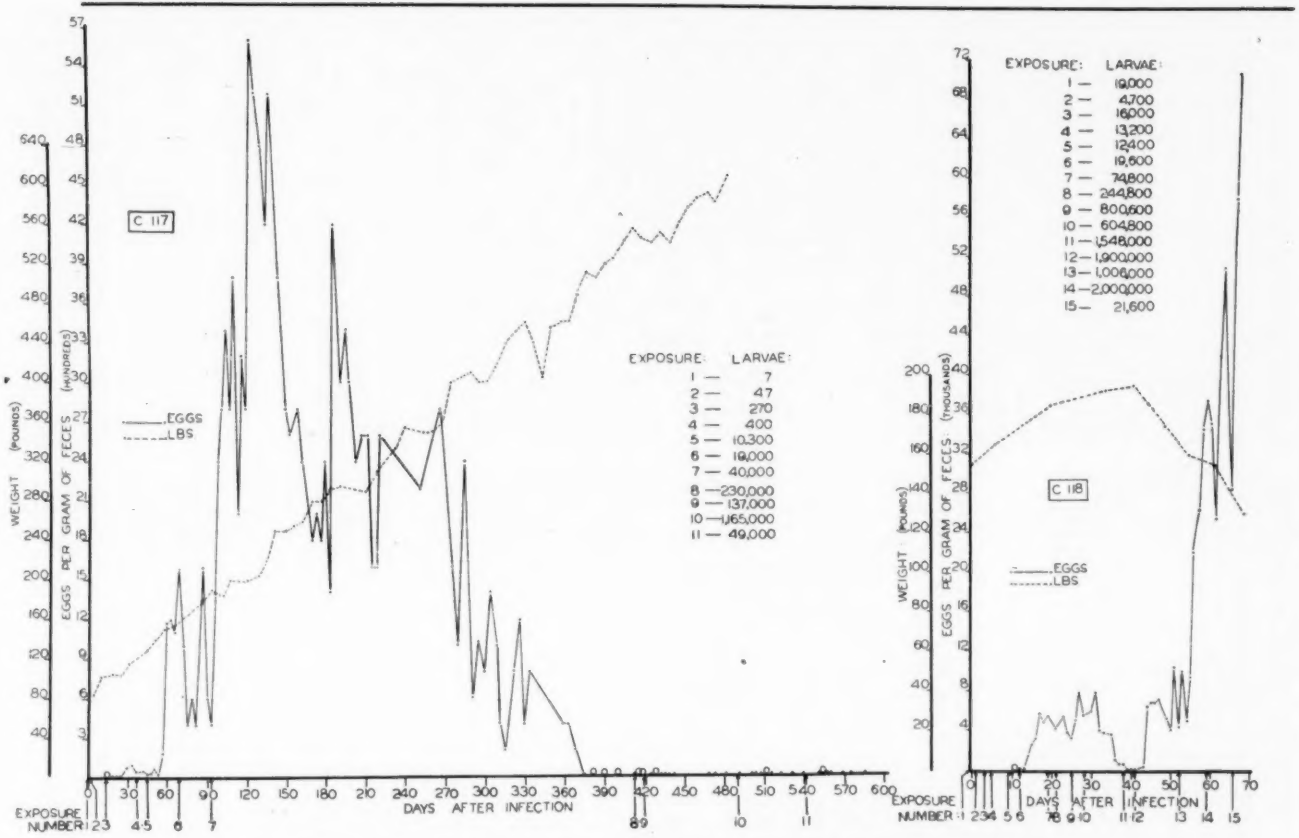

Fig. 1—Eggs per gram and weight curves for calves 117 and 118. Negative counts are shown as zeros on the base line. Solid points on the base line of the curve for 117 indicate a count of less than 25 eggs per gram, and for 118 a count of less than 200 eggs per gram.

sitic development was not accurately determined, but larvae were in the third or infective stage when isolated routinely from moss-feces mixtures seven to ten days after they had been prepared, having been held at room temperature. Larvae were collected from the moss by means of a large Baerman apparatus and were fed at once to calves or stored at room temperature in small jars of moss until needed.

The number of larvae was estimated by dilution counting before they were administered, except where the number given was small enough to permit counting of the total number.

All calves were infected by squirting the larvae into the back of the mouth by means of a dissecting blowpipe attached to a 5.0-cc. rubber bulb. The course of infection in each calf was followed by fecal egg counts. Large numbers of eggs were counted by the dilution technique and small numbers by the flotation technique as described in Stoll.[2]

Packed, red blood cell counts were made by the Wintrobe hematocrit method. Calves 117, 118, 119, 433, and 434 were weighed routinely, usually at weekly intervals. These weights are plotted on the figures of the EPG* counts of the respective calves to the point where there was no possibility of change due to infection.

At necropsy, complete examinations were made for evidence of gross pathology. Sections of the intestinal wall were fixed in Bouin's fixative or in 10 per cent formalin. From these, paraffin sections were made and stained with hematoxylin and eosin for use in the study of the histopathology. The number of worms present was approximated by examining aliquot parts of the contents and washings of the small intestine.

OBSERVATIONS AND DATA

Prepatent Period.—Table 1 shows that the prepatent period ranged from eleven to sixteen days in the 10 calves for which it is recorded. More calves first showed eggs in the feces on the twelfth day after infection than on any other. The calves ranged in age from 2½ to 12 months at the time of infection, and there was no relationship exhibited between the age of the animal and the prepatent period. It is interesting to note that calves 117 and 752 received a very small number of larvae in the initial dose and had the longest observed prepatent periods, sixteen and fifteen days, respectively. This apparently was due to such a small number of eggs in the feces that they were not detected earlier by the methods employed.

Patent Period.—Calf 117 was given approximately 70,000 larvae, in seven unequal doses, within the three months after the initial infection (table 1, fig. 1). It was then held without further exposure to infection to determine the length of time eggs would be passed in the feces. For 276 days after exposure 7, the EPG count was 200 or higher, and it dropped to negative for the first time 290 days after this infection.

Age Resistance.—In the report by Fourie (*loc. cit.*) on clinical parasitism in which the related species, *C. pectinata*, was found, 18-month-old animals were affected. This indicates the absence of a protective age resistance against that species at this age. To observe the effect of age, up to 12 months, on the development of resistance against *C. punctata*, calves 753 (12 months old) and 796 (5 months old) were infected with 100,000 larvae each. The larvae were from the same culture, and both calves had been previously uninfected with helminth parasites. At necropsy, eighteen and twenty-two days later, respectively, there were 60,000 worms present in the intestine of calf 753 and 44,400 in the intestine of calf 796 (60.0% and 44.4% development, respectively).

As a further observation, calf 754 (10 months old and also previously uninfected) received 26,000 larvae. The calf was killed seventeen days later, and 18,300 worms were present in the intestine. This represented a development of 70.4 per cent of the larvae administered to this calf.

Acquired Resistance.—It will be noted that the EPG count could not be accurately anticipated on the basis of the number of larvae administered, even with initial infections. Six of 7 calves given repeated infections showed an accumulation of parasites in the intestine as indicated by the rise in the EPG count with the repeated exposures. The exception was calf 989 (fig. 2) which was given 110,000 larvae initially. Subsequent exposures failed to cause an increase in the EPG count. The EPG count of this calf and of calves 753 and 796 (previously referred to) which also received large initial exposures was markedly lower than in calves 117, 433, and 434, all of which received smaller doses of larvae at the initial exposure.

Following the accumulation of parasites, there was a subsequent decrease in the EPG counts of these calves. This was taken as an indication that the host was acquiring a resistance and exhibiting a self-cure.

To observe the ability of the animals to resist reinfection or superimposed infection, 7 previously infected calves were given test doses of larvae varying in number from 30,000 to 1,165,000. Calves 117, 433, and 434 had apparently thrown off most of their worms, as indicated by the EPG counts shown on figures

*EPG will be used hereafter for "eggs per gram" of feces.

TABLE 1—Data on Experimental Infections with *Cooperia Punctata* in 11 Calves

Calf No.	Date of birth	Infective larvae given			Pre-patent period (days)	Days to necropsy		Worms recovered (No.)	Worms recovered (% of larvae given)
		Date	Number	Total No.		from first infection	from last infection		
117	7-20-42	9-29-42	7		16				
		9-30-42	47						
		10-12-42	270						
		11- 5-42	400						
		11-13-42	10,300						
		12- 8-42	19,000						
		12-31-42	40,000						
		11-17-43	230,000						
		11-24-43	137,000						
		2- 3-44	1,165,000						
		3-25-44	49,000	1,651,024		613	72	0	0.0
118	7-19-42	11-21-42	19,600		12				
		11-22-42	4,700						
		11-24-42	16,000						
		11-26-42	13,200						
		12- 1-42	12,400						
		12- 3-42	19,600						
		12-10-42	74,800						
		12-12-42	244,800						
		12-16-42	800,600						
		12-19-42	604,800						
		12-29-42	1,548,000						
		12-31-42	1,900,000						
		1-12-43	1,006,000						
		1-19-43	2,000,000						
		1-26-43	21,600	8,285,500		70	4	728,000	8.75
119	7-21-42	2- 3-44*	850,000		..				
		3-25-44	529,000	1,379,000		123	72	0	0.0
433	5-30-43	8- 3-43	25,000		12				
		8-13-43	158,000						
		12- 2-43	63,000						
		1-11-44	114,000						
		4-17-44	662,000	1,022,000		not killed	not killed	....	....
434	5-30-43	7- 9-43	9,000		12				
		7-10-43	15,000						
		8- 3-43	25,000						
		12- 2-43	30,000						
		1-11-44	60,000						
		4-17-44	933,000	1,072,000		not killed	not killed	....	....
752	4-10-45	8-10-45	55		15				
		9-18-45	67,000						
		10- 2-45	28,800						
		10-10-45	38,400						
		4- 8-46	100,000	234,255		261	17	30,140	12.9
753	4-17-45	4- 8-46	100,000	100,000	11	18	18	60,000	60.0
754	5- 2-45	3-13-46	26,000	26,000	11	17	17	18,300	70.4
796	10-29-45	4- 8-46	100,000	100,000	11	22	22	44,400	44.4
988	7-22-45	10-23-45	40,000		14				
		11-13-45	40,000						
		12- 4-45	40,000						
		12-23-45	40,000						
		2- 1-46	40,000						
		4- 8-46	100,000	300,000		189	22	2,000	0.6
989	8- 4-45	11- 1-45	110,000		12				
		12- 4-45	34,000						
		12-23-45	187,000						
		2- 1-46	210,000						
		4- 8-46	100,000	641,000		180	22	0	0.0

*This was the first time larvae were administered. The calf had previously picked up small numbers of larvae from the floor.

1 and 3, when these test exposures were given. These 3 calves were sufficiently resistant to protect them against any significant reinfection as shown by the continued low level egg counts. Following exposure 5 of calf 434, there was a slight rise in the EPG count, but it was not high enough to be considered significant. The marked rise in the EPG count for calf 433 on about the thirty-fifth day and for calf 434 on about the sixtieth day (fig. 3) may have been the result of an attack of shipping fever which caused both animals to go off feed and thus concentrated the eggs in a smaller quantity of feces.

Calf 117 was killed seventy-two days after the last exposure, at which time the egg count

was barely positive. There were so few worms present that none were found on microscopic examination of 5 per cent of the intestinal contents. Calves 433 and 434 were not killed but were transferred to another (unrelated) experiment.

Calf 752 (for which no figure is given) had shown a fairly constant EPG count, ranging from 50 to 150, for about six months after exposure 4, before a challenge dose of 100,000 larvae was given. At the same time, the previously uninfected calf 753 of about the same age, was given an equal number of larvae from the same culture. Seventeen days after this challenge dose was administered to calf 752, the EPG count had dropped to zero. On the following day, the calf was killed and 30,140 worms were found in the intestine. This represented only 12.9 per cent of the total number of larvae administered. If all these worms had developed from the last larvae given, it would have been a development of 30.1 per cent. The female worms collected from the intestine of calf 752 had few or no eggs in the uteri. As previously stated, there were 60,000 worms (60.0% development) recovered from calf 753, and the uteri of the females were filled with eggs.

Calf 988 had been given four equal doses of larvae at approximately twenty-day intervals, as the initial series of exposures (fig. 2). A fifth dose of the same number of larvae was administered forty days later. Following exposures 3, 4, and 5, there was a transitory decrease in the EPG count to negative or near negative. The count increased slightly about thirty days after the fifth exposure, with the calf's liberating approximately 150 EPG when the test exposure of 100,000 larvae was administered sixty-six days after exposure 5. Twelve days later, the EPG count had dropped to negative where it remained until the calf was killed on the seventeenth day after that exposure. On necropsy, 2,000 worms were re-

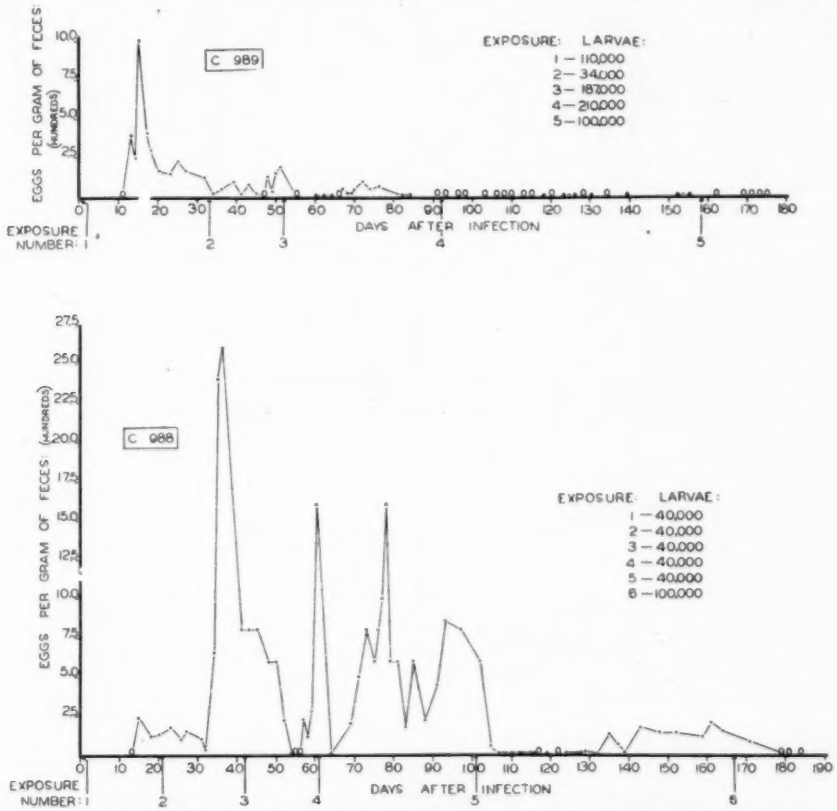

Fig. 2—Eggs per gram curves for calves 988 and 989. Negative counts are shown as zeros on the base line. Counts of less than 20 eggs per gram are shown as solid points on the base line.

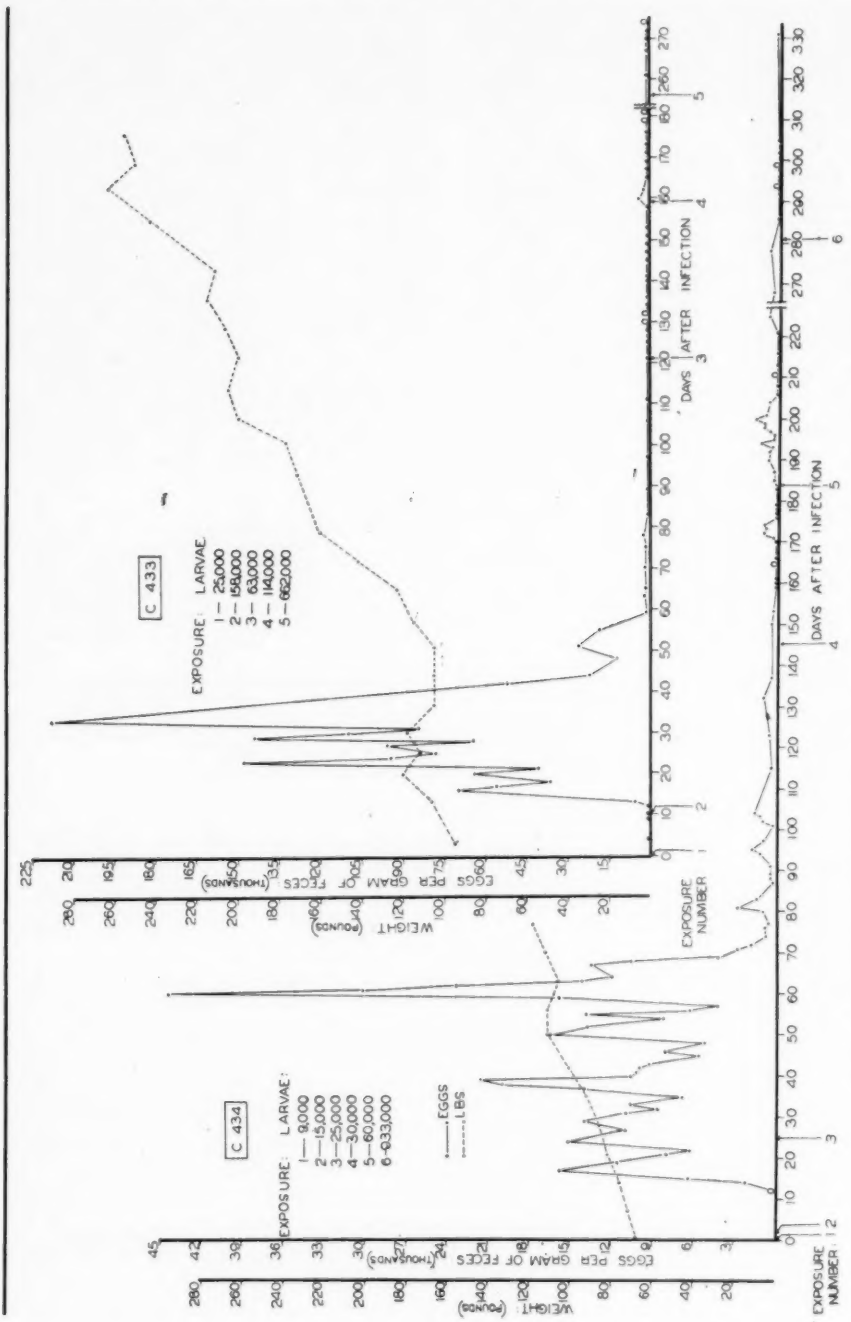

Fig. 3—Eggs per gram and weight curves for calves 433 and 434. Negative counts are shown as zeros on the base line, while solid points on the base line indicate a count of less than 100 eggs per gram.

covered from the intestine. The females had few or no eggs in the uteri. This represented 0.66 per cent of the total larvae fed to the calf, or a development of only 2.0 per cent if all the worms present had developed from the last exposure. This figure is much lower than the percentage of development from an equal number of larvae of the same culture in the previously uninfected calf 796 (44.4%). Female worms collected from calf 796 had uteri filled with eggs.

Calf 989, as has been previously stated, received 110,000 larvae in the initial infection, and then 34,000 larvae were given thirty-three days later (fig. 2). There was evidence of some drop in the EPG count about twelve to fourteen days later. On the fifty-second day, 187,000 larvae were administered with a resulting drop to negative and near negative for approximately sixteen days. Two other test doses (210,000 larvae on the ninety-second day and 100,000 on the one hundred fifty-eighth day) failed to reinfect the calf. The last exposure was with larvae from the same culture given at the same time as that for calves 752, 753, 796, and 988. Twenty-two days following the last exposure, calf 989 was killed, the EPG count having been negative for twenty days, and no worms were found in the intestine.

Data on another calf, 119, also illustrate the completeness of the immunity. In the earlier part of the experiments, this calf was held as the uninfected control, and it was planned that it would be kept parasite-free so that it could be infected initially at the time calf 117 was given its test doses. Unfortunately, the calf picked up a few larvae from the floor, being stalled adjacent to 117, but this did not occur until nine and a half months after 117 had started liberating eggs in the feces. Eggs were first found in the feces on Aug. 7, 1943, when the calf was slightly over 1 year old. The egg count varied from two to 40 EPG until in October, when the calf was placed in another barn of like construction on an unrelated experiment. It was returned to the original barn about Jan. 1, 1944, with the EPG count ranging from 0 to 25. On Feb. 3, 1944, a test dose of 850,000 larvae was administered (table 1). The EPG count dropped to negative and remained there for two months. Then there were several counts of 2 to 10 EPG. On March 25, 529,000 larvae were administered, with the egg count remaining unchanged. On necropsy, seventy-two days later, no worms were recovered from the intestine by means of the sampling technique.

Pathogenicity.—Of the 11 calves infected during the study, only 1 (118) showed significant clinical symptoms of infection. This calf was given large numbers of larvae at irregular short intervals (table 1 and fig. 1). As the result, the calf was overcome by these repeated doses of larvae. For three weeks after infection, it gained weight at approximately the same rate as did calf 119, as yet uninfected; but from the third to the sixth week it failed to show comparable gains. From the sixth week until death of the calf, it lost weight steadily until at the time of death it weighed 25 lb. less than when infected ten weeks earlier. Concurrent with the commencement of the downward curve in the weight, there was an appearance of soft feces, which soon developed into a marked diarrhea which persisted throughout the course of the infection. From the forty-fourth day of infection until death, there were intermittent periods of anorexia with the temperature remaining normal. With approaching death, the calf became visibly dehydrated and greatly emaciated. The weakness progressed to a point where the calf was unable to rise. When the temperature started declining and it was evident that the calf was in a moribund condition, it was destroyed so that the material might be examined before postmortem changes began.

Of special interest is the extremely high egg count of this calf. During the course of infection, the EPG count increased until on the day before death it had reached the peak of 70,600 (fig. 1). This is the highest EPG count the author has found reported for a nematode infection in domestic animals. Of course the calf had been off feed for three days, which would tend to increase the EPG count by decreasing the fecal volume output. However, the very profuse diarrhea would have at least partially compensated for that. By the sampling technique, it was determined that there were approximately 728,000 worms in the small intestine at necropsy (table 1).

Monnig[24] states that the Cooperia suck blood and in heavy infections produce a more marked anemia than is seen in trichostrongylosis. Calf 118 failed to show any clinical evidence of anemia, the volume of packed, red blood cells varying from 31.0 per cent to 37.0 per cent, which is within the normal range, except for the reading on the sixty-ninth day when it had increased to 42.0 per cent due to dehydration.

Calf 117 harbored parasites for approximately 600 days and also failed to show any evidence of anemia or a decrease in the packed, red cell volume. The packed cell volume for this calf ranged from 29.0 per cent to 35.5 per cent.

Occasional diarrhea was the only symptom manifested by the other infected calves. It is significant that a transitory diarrhea often followed the administration of test doses of larvae to the resistant animals.

Gross Pathology.—The most marked lesions

were found in the small intestine of calf 118. There were innumerable petechial and ecchymotic hemorrhages on the surface of the mucosa throughout the first 10 ft. of the intestine. In one place within this area, there was a single diffuse hemorrhage approximately ⅝ in., in diameter. Posteriorly from this first 10 ft. the hemorrhages gradually decreased, and the lower half of the intestine appeared normal except for a small amount of catarrhal exudate.

The surface of the mucosa in the upper part of the intestine presented an unusual greyish white appearance due to a very fine, lace-like fibrino-necrotic exudate. The wall of the intestine was slightly thickened. There was a varying amount of catarrhal exudate throughout most of the small intestine.

Fig. 4—Section of the upper part of the small intestine of calf 754 showing lymphocytic infiltration and fragmentation of epithelium, with an adult worm lying within this area of fragmented cells and exudate. x 110.

The parasites were more numerous in the first 30 ft. of the intestine but were present throughout, and some were found in the large intestine. Due to the absence of lesions in the lower part of the small intestine, it seems logical to assume that the worms found there, and in the large intestine, were swept there by the increased peristalsis. The mesenteric lymph nodes were slightly edematous. The gall bladder was greatly enlarged, probably due to closure or partial closure of the opening of the bile duct into the intestine by the inflammatory exudate.

All the other calves showed essentially the same type of enteritis, but less severe. Calves 117 and 119, which were killed about two months after their last exposure (which failed to establish), still showed innumerable petechial hemorrhages. As in calf 118, the hemorrhages were more numerous in the duodenum and in all calves appeared to be more prevalent on the tips of the folds. The intestines of calves 753, 754, and 796, all of which received only one dose of larvae (table 1), presented the characteristic petechial hemorrhages. However, they were not as numerous nor did they extend as far down the intestine as in calves 118 and 117. The greyish white exudate on the surface of the mucosa was also present in some degree in all calves examined.

Histopathology.—The mucosa and submucosa showed marked lymphocytic infiltration, especially in the lamina propria of the mucosa. This was characteristic in all the calves on which necropsy was performed, varying some in degree. Many eosinophilic leucocytes were present in most sections of the intestines, in those from calves infected only once (calves 753, 754, and 796) as well as the others. There was an extensive fragmentation of the epithelium of the mucosa, especially of the upper part of the small intestine (fig. 4). The tissue debris comprised a part of the catarrhal exudate present on the surface. The adult parasites were commonly found within or under this exudate. There was marked hyperplasia of the lymph follicles, and the blood vessels in all sections showed congestion.

As was expected, the intestine of calf 118 showed the most marked microscopic changes. The submucosa of the upper part of the intestine was thickened, due partially to an infiltration of serous exudate. In many intercellular spaces of this layer, there were large numbers of free erythrocytes. At places, the mucosa showed extensive degeneration and the

presence of a thick fibrino-necrotic exudate. Adult worms and larvae were found in the lower portions of the glandular crypts and even penetrated to the submucosa and the serosa in the upper part of the intestine of this calf. These lesions were similar to the ones described by Hung (*loc. cit.*), except that the parasites had penetrated deeper than had those he described. Some of the adults were encysted in the serosa which showed marked thickening (fig. 5). The tissue around these encysted worms in the serosa and submucosa was partially necrotic.

Many erythrocytes could be seen in the digestive tract of some adults found in the lower portions of the crypts. Hung found the same and supposed that the parasites were blood suckers. No erythrocytes were found in the digestive systems of worms on the surface of the mucosa. It is interesting to note that neither larvae nor adults were found to have penetrated within the tissue proper in the calves that were less heavily infected. In this connection, the calves from which Ransom and Hung (*loc. cit.*) secured the material for their study were apparently heavily parasitized (as indicated in their discussion). In the intestinal wall of calf 118, there were found no embryonated eggs nor first stage larvae as were found by Hung.

DISCUSSION

On the basis of one controlled observation, there was no evidence of a protective age resistance against *C. punctata* in calves up to 12 months old. Data presented show that the patent period of the infection may be as long as nine months. Repeated exposure to infective larvae results in a much shorter course of infection which appears to be the result of an active immunity developed by the host.

From the data obtained, it is believed that this acquired immunity is essentially the same as that previously demonstrated by other workers. Stoll[15] reported the development of an acquired resistance to the stomach worm, *Haemonchus contortus*, in 2 lambs. In this he showed that, under conditions permitting natural reinfection, there was first an accumulation of a parasite load and then a self-cure which resulted in the expulsion of the worms and subsequently protected the animals against any significant amount of further infection with the parasite. Mayhew[16] reported the development of resistance against *H. contortus* in calves.

Similar results have been demonstrated in experiments with *Ancylostoma caninum* by McCoy[17] and Foster.[18] Sarles[19] was able to demonstrate an acquired immunity to *Trichostrongylus calcaratus* of the rabbit, in which a similar self-cure reaction was exhibited following the administration of six to eight weekly doses of larvae. In contrast to this sudden loss of infection following repeated doses was the long patent period of forty-nine weeks in a rabbit given a single dose of larvae.

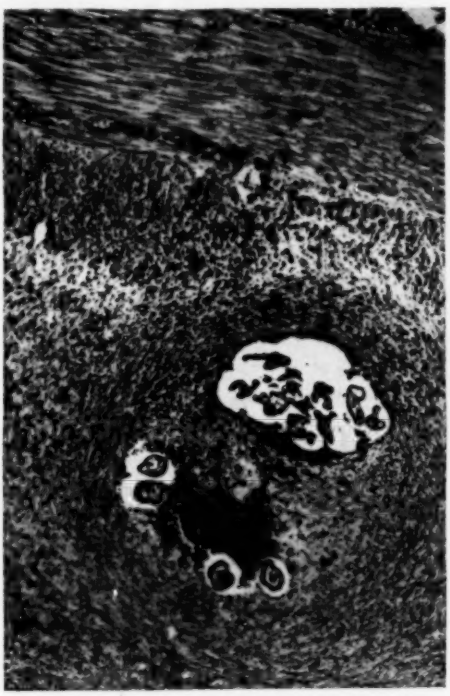

Fig. 5—Section of the upper part of the small intestine of calf 118 showing encysted adult worms in the thickened serosa. x 68.

Chandler,[20] Graham,[21] and Schwartz, Alicata, and Lucker[22] demonstrated the development of a similar acquired resistance by rats against *Nippostrongylus muris*, as did Sheldon[23] in rats against *Strongyloides ratti*. Mayhew[24] produced an immunity to the nodular worm and hookworm in 3 calves, in which case the resistance gave evidence of lacking permanency.

In all the calves that harbored the parasite for as long as three months, without

regard to the intervals between exposures or the number of larvae given (within the ranges used in these experiments), an immunity developed that was sufficient to protect the animals against significant reinfection. This was true even when very large numbers of larvae were given as test doses.

Even in calf 118, there was evidence of a beginning self-cure. From the thirty-first to the fortieth day there was a marked drop in the EPG count to near negative, as if the animal were developing a resistance. However, it seems reasonable to conclude that the repeated heavy exposure overwhelmed the host before sufficient resistance could be developed.

The administration of larvae to resistant calves which were liberating eggs in the feces resulted, in most instances, in a drop in the EPG count, often to negative or near negative. Such a drop is seen following exposure 5 of calf 988 (fig. 2). There is some evidence that the suppression of egg production on the part of the female worms is one of the first manifestations of a developing resistance of the host. The decrease in the EPG count may be only temporary if the immune response has not been stimulated to the point where the worms are expelled. Mature female worms, containing few or no eggs, found in the intestines of calves 988 and 752, following such a drop in the egg count, supports this supposition.

That the ingestion of larvae by an infected animal is associated with the stimulation of resistance is substantiated by the previously stated observation that repeated exposure results in a much shorter patent period.

The resistance of calf 119 to reinfection or superimposed infection was as complete as in any calf having had a much heavier infection. However, the possibility of some age resistance cannot be eliminated here, as this animal was nearly 2 years old at the time of challenge exposure.

As previously stated, *C. punctata* has been incriminated as a blood sucker. The only evidence observed in this study that could possibly lend support to that view was the presence of erythrocytes in the digestive tract of worms lying in the lower portion of the intestinal crypts. The author is of the opinion that these erythrocytes had been liberated by the rupture of capillaries or by the phenomenon of diapedesis and were engulfed somewhat accidently by the worms as they fed on tissue debris. Supporting this view is the fact that many extravascular erythrocytes were observed in the submucosa of calf 118. It is inconceivable that the worms feed on blood and still produce no evidence of anemia in a calf harboring over 700,000 of them and dying as the result of this massive infection.

Inasmuch as no larvae or adults were found to have penetrated the intestinal wall of any calf other than 118, it appears that the worms do not migrate in the wall of the intestine except in heavy infections. But there might be other (as yet undetermined) factors involved. In this connection, it is interesting to note the statement by Foster[25] to the effect that it is possible that ingested larvae (of the dog hookworm) migrate in greater numbers in resistant animals.

SUMMARY

A total of 11 calves were artificially infected with *Cooperia punctata*. The prepatent period ranged from eleven to sixteen days, with age (at least up to 1 year) having no effect on the time. The patent period was observed to be as long as nine months but was consistently shortened by repeated exposure of the host.

In one controlled test, there was no evidence of a protective age resistance produced in calves up to 12 months old. Following initial and repeated exposure, the parasite load was built up within the host, with a succeeding self-cure, enabling the host to expel the parasites.

In all of 7 calves which harbored the parasite for as long as three months, an active immunity was demonstrated which was sufficient to protect the host against significant reinfection or superimposed infection, even when large numbers of larvae were given.

The parasite was found to be pathogenic. One calf showed clinical symptoms including diarrhea, anorexia, dehydration, and progressive emaciation leading to death. There was no evidence of anemia in any of the infected calves.

The gross lesions were largely confined to the duodenum and consisted of a catarrhal inflammation with a fine fibrinonecrotic exudate, hemorrhages, and a thick-

ening of the intestinal wall. Microscopically, there was a leucocytic and serous infiltration of the intestinal wall, especially of the submucosa. Localized degeneration with marked fragmentation of the mucosa was evident. The worms were found principally in contact with the surface but in 1 calf had penetrated even to the serosa.

The author is indebted to Dr. D. A. Porter of the Regional Animal Disease Research Laboratory for invaluable assistance in outlining the study. Appreciation is also expressed to Dr. B. T. Simms, then director of the Regional Animal Disease Research Laboratory and now chief of the Bureau of Animal Industry, for his helpful suggestions throughout the study.

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Statistical Considerations of Controlled Experiments in Brucellosis

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ONE OF THE major questions to be answered in any experiment in which an attempt is made to measure the efficiency of a treatment of the experimental material is that of the difference which is displayed by the treated animals in comparison with the response of the untreated controls. If the data are expressed in percentages, as the proportion of animals aborting from brucellosis in cattle, they are just as amenable to statistical treatment as if other kinds of measurements had been made. It is well known however that, if only a small number of individuals is involved in an experiment, the outcome of which is expressed in percentages, a marked difference between the response of those treated and the response of the controls is required, in order to be reasonably certain that the actual differences are statistically significant and not simply errors of random sampling.

Many workers prefer not to compare the percentages of abortion or infection in lots of animals involving small numbers, but rather, prefer to compare the actual numbers in the groups according to their reactions. For example, if there were two lots of 10 animals each, and in one lot, 9 of the 10 (90%) animals aborted as the result of an exposure to *Brucella abortus* and, in the other, only 7 (70%) abortions in 10 occurred, would this difference (20%) be significant? In other words, how much variation in the actual results in tests on a small number of animals may be expected from random sampling alone, assuming that all experimental conditions were constant? This point must be considered before any satisfactory measurement of the influence of various other factors is possible. The extent of this variation in groups with different numbers is presented below.

Obviously, small lots of experimental animals are usual in studies of bovine brucellosis. If then, the results of induced infections from several laboratories, or of induced infections at different times at the same laboratory are grouped, the question arises whether the average percentage of abortions of the total is reasonably typical of all the tests. That is, are the variations among the percentages of abortions in the different tests so great that the average percentage cannot be considered as being uniform from test to test? Many variables could conceivably be responsible for variation in the results: (1) dosage of the organisms, (2) virulence of the organisms, (3) stage of gestation when the exposure was made, etc. (An example of the kind of statistical analysis required for such data is that used by McPhee,[22] who found the data from an experimental herd of swine to be homogeneous as to sex ratio.)

The authors believe that this is an opportune time to summarize the various reports dealing with the incidence of abortions among normal cattle, *i.e.*, those which have not been known to have had a previous infection of brucellosis, induced by artificial exposure and to determine by a statistical analysis whether the observed variations in results are to be expected as a result of normal variations, *i.e.*, random sampling. If the variations in the respective proportions of aborting animals in the different trials exceed those expected as the result of chance, further standardization of technique might well make future results from different laboratories more comparable. Also, it appears desirable to re-emphasize some of the pitfalls into which the unwary may fall in a comparison of different experiments in which only a few animals are involved. This applies to the results of such varying treatments as differences in dosage, duration of acquired immunity through vaccination, etc.

METHODS

A compilation has been made of the reports in the literature of the reactions of normal animals to induced infections with *Br. abortus*. These are summarized in table 1. Except in two trials (6, 12), only those tests are included

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in which the animals were exposed by way of the conjunctiva to a dose of at least a million organisms, usually many-fold larger. The animals are grouped in the table according to whether they aborted, produced living but weak calves (a) with or (b) without the recovery of the organism, or produced normal calves at full term. For purposes of statistical comparison, these four groups are further combined into two categories, "resistant" and "susceptible." Those animals which produced living but weak calves are combined with those which aborted, with isolation of the organism in each case, and are called the "susceptibles." The "resistant" group is comprised of (a) those animals which produced living but weak calves within a month of the normal gestation period of 282 days (Lush[20] gives a standard deviation of five days) but with no isolation of the organism and (b) all individuals which calved normally, whether or not organisms were recovered. This grouping** is admittedly arbitrary, but allows the analysis of contrasting groups—those aborting and those calving normally.

In the 36 tests summarized in table 1, there was a total of 353 normal cows exposed to *Br. abortus*. Of these 303 (85.84%) aborted, and 50 (14.16%) calved normally. The lowest percentage of abortions was in one trial of 9 animals,[14] in which only 3 aborted, or a rate of 33.3 per cent for this small sample, contrasted with many tests in which all the animals were susceptible, *i.e.*, all aborted. The first step in analyzing this summary is to determine whether the average percentage (85.84) for these 36 trials is representative of the different tests. That is, is the variation in the various tests no greater than that expected by chance?

The proportions of abortions among these 36 different experiments are distributed as follows: There was one lot with an abortion rate between 31 and 40 per cent, inclusively, one between 41 and 50 per cent, one between 51 and 60 per cent, five between 61 and 70 per cent, two between 71 and 80 per cent, nine between 81 and 90 per cent, and 17 between 91 and 100 per cent. As is stated above, the question to be answered is whether this distribution of percentages may normally be expected if the underlying probability of abortion in each experiment were 85.84 per cent.

**A division could have been made equally well according to whether or not the animals were infected. Such a grouping would have meant some changes in tables 1 and 3, but would not have affected significantly the deductions from the analysis of either table. As may be noted in these tables, the number of animals in each experiment is designated which calved normally but from which *Br. abortus* was recovered.

TABLE 1—A Summary of the Results of Conjunctival Exposure of Normal Cattle to Brucella Abortus

Abortions	L-W infected	L-W Noninfected	Normal calving	Totals	Susceptible (%)	References
7	0	0	1*	8	87.50	Buck and Creech[6]
3	0	0	2	5	60.0	Buck[4]
7	0	0	1*	8	87.50	Cotton, *et al.*[5a]
5	0	0	2	7	71.43	Cotton[7]
7	1	0	1	9	88.89	Cotton, *et al.*[5b]
8	0	0	0	8	100.0	Cotton, *et al.*[9]
7	0	0	4	11	63.63	Cotton, *et al.*[9]
7	3	0	6**	16	62.50	Cotton, *et al.*[10]
10	0	0	0	10	100.0	Report, Chief, BAI[25]
2	0	0	1	3	66.67	Delez[12]
5	1	0	3	9	66.67	Report, Chief, BAI[26]
5	0	0	1	6	83.33	Haring & Traum[13]
7	4	0	0	11	100.0	Haring & Traum[13]
7	0	0	0	7	100.0	Haring & Traum[13]
4	0	0	0	4	100.0	Report, Chief, BAI[27]
7	0	0	2**	9	77.78	McEwen, *et al.*[21]
8	1	0	1	10	90.0	McEwen, *et al.*[21]
3	2	1	4**	10	50.0	McEwen, *et al.*[21]
3	1	0	0	4	100.0	Delez[12]
19	0	0	0	19	100.0	Crawford & Beach[11]
17	3	0	0	20	100.0	Crawford & Beach[11]
17	2	0	0	19	100.0	Crawford & Beach[11]
16	2	0	1*	19	94.74	Crawford & Beach[11]
8***	1	0	5	14	64.28	Beach, *et al.*[2]
19	0	0	4**	23	82.61	Beach, *et al.*[2]
3	1	0	0	4	100.0	Hutchings[19]
5	0	0	1	6	83.33	Hutchings[19]
0	3	1	5	9	33.33	Edwards, *et al.*[14]
7	2	0	0	9	100.0	Edwards, *et al.*[14]
6	2	0	1*	9	88.89	Edwards, *et al.*[15]
9	1	0	0	10	100.0	Edwards, *et al.*[15]
10	0	0	0	10	100.0	Beach, *et al.*[1]
8	0	0	0	8	100.0	Report, Chief, BAI[28]
5	0	0	0	5	100.0	Report, Chief, BAI[28]
6	1	0	1*	8	87.50	Paterson[23]
5	0	0	1	6	83.33	Paterson[23]

L-W indicates living-weak.
*Brucella organisms recovered from 1 animal.
**Brucella organisms recovered from 3 animals.
***Three additional animals aborted, but the organisms were not recovered.
Dosages were a million or more organisms.

The method of performing the computations to determine this point is explained elsewhere.[26] Briefly, the product of the variations in the proportion of abortions observed in each subset (trial), weighted according to the number of animals used, is compared with the variation expected if the probability of abortion in each subset were the average (85.84%) of all the tests. The ratio existing between these two figures is $\frac{115032.29}{1215.8098}$, or 94.61, which represents the value of X^2 (chi-square). From a table of the value of X^2, it can be seen that for 35 subsets (n–1) this value would be obtained by chance alone much less often than once in 100 trials (p is less than .01). Hence, the variations noted among the various lots are too large to be explained by random sampling. One must then look for an explanation of this variation from the average percentage (85.84) of abortions to factors within each of the various trials. (In biologic material, odds less than 1 in 20 are usually accepted as indicating significance. Therefore, odds less than 1 in 100 can be regarded as highly significant. Only odds less than 1 in 100 will be considered significant in this paper.)

TABLE 2—The Proportion of Abortions Expected in n Trials of 10 Animals Each when the Probability of Abortion in a Single Trial Is p†

The number of animals in each lot is 10;
p = probability of abortions,
q = probability of normal calving

Abortions	Normal calvings	p=0.5 q=0.5	p=0.6 q=0.4	p=0.7 q=0.3	p=0.8 q=0.2	p=0.9 q=0.1
10	0	.001	.006	.028	.107	.349
9	1	.009	.040	.121	.268	.387
8	2	.043	.121	.233	.302	.194
7	3	.117	.215	.267	.201	.057
6	4	.205	.251	.200	.088	.011
5	5	.246	.201	.103	.026	.001
4	6	.205	.111	.037	.006	.000
3	7	.117	.042	.009	.001	.000
2	8	.044	.011	.001	.000	.000
1	9	.009	.002	.000	.000	.000
0	10	.001	.000	.000	.000	.000

The digits represent the proportion of abortions expected per 100 trials of 10 animals each, for the respective probabilities (p) as given.

†We are indebted for the preparation of this and other tables to Professor K. J. Arnold of the Department of Mathematics, University of Wisconsin. Mimeographed copies of this table, and of comparable tables involving 15, 20, 25, 30, 40, and 50 animals in a trial may be obtained upon request.

It was suggested by Webster[27] that the reactions of laboratory animals to experimental infections were conditioned by three main variables, assuming that all other procedures relating to the care, nutrition, and management of the animals were reasonably uniform. These variables are (a) dosage, or the number of organisms administered, (b) virulence of the organisms, and (c) individuality of the host animals. The authors believe that these variables are of major importance in both natural and induced outbreaks of brucellosis. But to these three variables in the different trials cited in table 1 must be added the differing environmental conditions which undoubtedly existed in the several laboratories, such as the nutrition of the animals, time in the gestation period when exposed, etc.

The dosages of the organisms certainly varied greatly in the 36 trials, and it is reasonable to believe that there also was variation in the virulence of the cultures used in the exposures. In 16 of the 36 trials, all the animals aborted, a result which could sometimes be produced if overwhelming numbers were administered. However, it is not the purpose of this paper to attempt to assess the conditions that produced the variations among the various tests.

As was stated earlier, one test[14] of 9 animals resulted in only 3 abortions, or at the rate of 33.3 per cent susceptibility of this lot; in another test[21] of 10 animals only 5 aborted. Would these proportions of abortions be expected if the conditions were such that the probability of abortions was 80 per cent in each test? In other words, if 10 animals are exposed and 80 per cent of these are expected to abort (p = .8), would the abortion by only 5 animals mean that the dosage was too small, or, would the abortion by all 10 animals mean that the infection was too severe?

A very useful table was compiled by Pearl[28] in which are listed the chances of success of an event (as an abortion) happening among 10 principals (as cattle) if the probability for it is, respectively, 0.5, 0.6, 0.7, 0.8, and 0.9. For purposes of illustration a comparable table is given in table 2.

Referring to the values in table 2, it may be seen that if the underlying probability (p) of

TABLE 3—The Results of Conjunctival Exposure of Normal Cattle with Varying Doses of Brucella Abortus, Strain 544

Dosages	Abortions	Normal calvings	Total	Reference
$1,460 \times 10^8$	7	2	9	McEwen, *et al.*[21]
150×10^8	9	0	9	Edwards, *et al.*[14]
150×10^8	10	0	10	Edwards, *et al.*[15]
$1,460 \times 10^4$	9	1	10	McEwen, *et al.*[21]
15×10^8	8	1	9	Edwards, *et al.*[15]
$1,675 \times 10^4$	7	1	8	Paterson[23]
14×10^8	5	1	6	Paterson[23]
$1,460 \times 10^3$	5	5	10	McEwen, *et al.*[21]
10^8	3	6	9	Edwards, *et al.*[14]
$1,460 \times 10^2$	3	6	9	McEwen, *et al.*[21]
1,460	0	9	9	McEwen, *et al.*[21]

abortions in cattle is 0.8 (under column p = .8, q = .2), on the basis of random sampling 4 abortions in 10 animals would be expected once (.006) in 100 trials; 5 abortions in 10 would be expected three times (.026) in 100; 6 abortions in 10, nine times (.088) in 100; and 7, 8, 9, and 10 abortions in 10 animals would occur respectively, 20, 30, 27, and 11 times in 100 trials. If p = 0.8, 3 or less abortions in 10 animals are rarely to be expected—less than once in 100 trials. Therefore, if only 3 abortions were produced in a group of 10 individuals, and previous experience indicated that the expectation of abortion under the same conditions was 80 per cent, the experimenter would have grounds for believing that the virulence of the organism had been reduced, or that some other unexpected variable had been introduced.‡ But the abortion of 4 to 10 animals could occur and still be within the limits of normal expectation.

From this table, it can also be noted that 9 abortions in 10 animals would normally be expected once (.009) in 100 trials of 10 animals each if the probability of abortion were 0.5; 4 times (.04) in 100 trials if the probability were 0.6; and 12, 27, and 39 times in 100 trials if the respective probabilities were 0.7, 0.8, and 0.9. In other words, if 9 abortions occurred in a test on 10 animals, this number could be obtained if the probability of abortion were 0.9, 0.8, or 0.7. It could occur less frequently, but still within the limits of random sampling, if the probability of abortion were 0.6, and could occur rarely even if the probability were as low as 0.5.

These examples show that percentages (probabilities) obtained in the use of small numbers should be treated with extreme caution. In comparing the differences between any two groups, each involving numbers as small as 10 animals, a difference as great as 40 per cent and occasionally even of 50 per cent, may not be statistically significant.

Dosage.—A question of primary importance to all investigators in brucellosis is that of an adequate but not overwhelming number of organisms for an artificial exposure. A limited amount of pertinent information is available in the literature as to the effect of varying numbers of the organism on the abortion rate in normal animals, when administered conjunctivally. McEwen, *et al.*[21] used five graded doses of a particular culture of *Br. abortus* (544) on respective lots of 9 or 10 animals each. Other tests of different dosages with the same strain of the pathogen were made by Edwards, *et al*,[14, 15] and by Paterson.[23] For ease in comparison, the results of the several tests are combined in table 3.

It will be noted, that the heaviest dose, over a billion organisms or $1,460 \times 10^6$, caused 7 of 9 animals to abort in one test[21] in contrast with about one-tenth this number of organisms which produced abortions in each of 9 animals in one trial,[14] and in each of 10 in another.[15] From these tests, it seems clear that an excessive dose of a particular strain of the organism need not cause all normal animals to abort. The abortion of all the animals in each of the two lots exposed to 150 million organisms would, therefore, appear to be a sampling due to chance, with the underlying probability (p) of abortions somewhere between 0.7 and 0.95 (*see* table 2).

There were four trials in which the dose was approximately 15 million organisms. In each of these, all but 1 animal aborted—9 abortions in 10 animals,[21] 8 in 9 animals,[15] 7 in 8, and 5 in 6 animals,[23] respectively. Combining these tests, it is seen that this dosage produced 29 abortions in 33 animals. These results are not significantly different from those obtained following the use of 10 times the number of organisms (150 million), nor from that of the single lot given over a billion organisms. In other words, if there is a difference in the proportions of abortions expected from a dose of 15 million or more organisms, as compared to 150 million, many more animals than have been used in these tests will be required to establish the difference.

When the dosage was further reduced to approximately a million organisms, in two different trials, a statistically significant reduction in the rate of infection was obtained. In one of these tests,[21] 5 of 10 animals aborted, in the other,[14] 3 abortions occurred in 9 animals. These proportions *would* be possible if the probability (p) of abortion were 0.6, but if p = 0.5 or less, the chances of their occurrence would be greater (*see* table 2). Less than a million organisms ($1,460 \times 10^3$) caused 3 abortions in 9 animals, and slightly over a thousand (1,460) caused no abortions in 9 individuals.

Comparable doses of this strain (544) of *Br. abortus* in the different laboratories produced results no different than would be expected if the tests had been performed in the same laboratory. Another example of the uniformity of activity of another culture of *Br. abortus* in different laboratories is that of strain 2308 in trials on two lots of 8 and 5 animals, respectively, in one laboratory,[23] and on 10 animals at another.[1] The number of organisms given each group was approximately 30 million, and each of the animals in the three lots aborted. Seemingly, this strain is highly

‡The authors are well aware that the usual method of statistical analysis would be to compare the percentage of abortions observed in a particular trial with the proportion of abortions expected. Unfortunately, at the present writing, the proportion of abortions to be expected with a given technique is practically unknown because of the wide variation in technique employed in the various laboratories. When sufficient data have accumulated to be reasonably certain of the expected proportion of abortions with a given technique, the above type of analysis should be employed.

virulent for cattle, perhaps even more so than the strain cited previously (544).

The examples given are illustrative of possible differences in virulence between two strains of *Br. abortus*. Assuming that such differences in virulence are a reality, it is obvious that a comparison of dosages of different strains of the organism might well be misleading, unless the virulence of the respective strains were comparable. For example, at one exposure of 14 normal animals of herd 2 at this laboratory,[3] over 2 billion viable organisms were administered by way of the conjunctiva. Nine of these 14 aborted; the other 5 produced normal calves. In contrast, 30 to 33 million organisms of strain 2308 caused each of 10 animals in herd 4 to abort.[1] The differences in the abortions in these two groups are not statistically significant, but the trend is quite definite. Some factor or factors made the exposure somewhat more severe in herd 4. The cows in herd 2 were exposed between the first and fourth month of pregnancy; those of herd 4 between the fourth and sixth month. Unless cattle are more susceptible to brucellosis during the later stages of gestation than in early stages, it appears that differences in the virulence of the pathogen used in these two tests may account for at least a part of the probable differences in rates of abortions.

The Uniformity of the Results Obtained by Calfhood Vaccination with Strain 19.—The results of exposing animals vaccinated as calves with strain 19 in their first pregnancy and under controlled conditions are summarized in table 4. Using the same method for testing the homogeneity of these data as was employed in analyzing the data of table 1, it appears that the 89.13 per cent of normal calvings observed in these 7 trials may be taken as the underlying probability in each sample. The value of X^2 in these calculations is 11.42395, which is well within that expected in random sampling (p is more than 0.05 and less than 0.10) for this number of trials.

The base line for a comparison of animals protected against brucellosis by vaccination or otherwise, is the rate of abortion expected in normal animals under comparable conditions. In the tests on 353 animals cited earlier, this rate was about 86 per cent, with a considerable range above or below. In contrast, the observed abortions among cattle vaccinated as calves and exposed by the conjunctival route during their first gestation period were 5 in 46 individuals (*see* table 3). There is no question but that the difference between these two sets of figures is highly significant and that calfhood vaccination with strain 19 conferred protection upon the majority of these 46 animals. (Also, trials with natural exposure[5] and field trials have added convincing evidence of the efficacy of calfhood vaccination with strain 19 in the protection of animals against *Br. abortus*, especially in the first calving.)

TABLE 4—The Reactions of Animals Vaccinated (with Strain 19) as Calves to Conjunctival Exposure with Brucella Abortus in the First Gestation Period

Abortions	L-W infected	L-W Noninfected	Normal calving	Total	Susceptible (%)	References
0	0	0	3	3	0	Buck[4]
0	0	0	9*	9	0	Cotton, *et al.*[10]
2	0	0	2	4	50.0	Haring & Traum[18]
0	0	0	2	2	0	Buck & Cotton[5]
0	2	0	8	10	20.0	Report, Chief, BAI[25]
0	0	1	13**	14	0	Report, Chief, BAI[26]
0	1	0	3	4	25.0	Report, Chief, BAI[27]

*Brucella organisms recovered from 1 animal.
**Brucella organisms recovered from 2 animals.
The dosages were a million or more organisms.

Duration of Acquired Immunity Following Vaccination.—As stated previously, the majority of cows which have been vaccinated as calves with strain 19 of *Br. abortus* will be protected during their first pregnancy. It is not clear, however, how long this acquired immunity will endure. Very little information is available at present concerning the duration of this acquired resistance beyond the first gestation.

Haring and Traum[18] reported abortions in 2 of 8 animals whose immunity was challenged during the second gestation following vaccination as adults, and Delez[13] noted 3 abortions in 9 animals following induced exposure in the second gestation following vaccination as calves. Results quite at variance with those expected in the first gestation, of animals vaccinated as calves, were obtained in this laboratory[1] subsequent to exposure during the third gestation. Of 23 cows vaccinated as calves, 9 calved normally, and 14 aborted or produced living but weak calves from which the organisms were recovered. When these results are compared statistically with those obtained in other laboratories by exposure in the first gestation period, as summarized in table 3, the differences are highly significant. That is, beyond question these animals did not show the same resistance to an induced infection in the third gestation that would have been expected had they been exposed during the first gestation. On the other hand, tests on only 23 animals represent

a relatively small sample from which to argue that this same decrease in the proportion of resistance will be typical of the thousands of animals vaccinated as calves. Reference to a probability table, with 20 or 25 animals at test, shows that 9 normal calvings in 23 animals would be expected infrequently if the probability of abortion were either 0.7, 0.6, or 0.5, and might occur even less frequently if the probability were 0.8. In other words, although the results with this small number of animals were 14 abortions and 9 normal calvings in 23, approximately 61 per cent abortions, these proportions might occur if the probability of abortion were 0.5, 0.7 or (occasionally) 0.8, rather than the percentage (0.61) observed.

The results from this laboratory are quite at odds with those obtained in the Bureau of Animal Industry.[28] In their hands, animals vaccinated as calves and exposed by way of the conjunctiva in the fourth and fifth pregnancy had practically the same proportion of resistant individuals as is expected during the first gestation period. Of 19 principals in the fourth pregnancy, 16 produced normal calves, as did 9 of 10 animals in the fifth pregnancy. The 16 normal calvings in the fourth pregnancy is 84.2 per cent of 19 animals. This observed proportion of normal calvings (17 normal calves and 3 abortions in 20 animals) would occur 19 times in 100 trials if the probability (p) of normal calving were 0.9, 20 times in 100 trials if $p = 0.8$, 7 times in 100 trials if $p = 0.7$, and once in 100 trials if $p = 0.6$ (these figures are taken from a table with 20 animals at test). In other words, although the actual percentage of normal calvings was 0.84 in this small number of animals, the same number of normal calvings could occur if the probability (percentage) were 0.9, or 0.7, and occasionally even if it were only 0.6.

The number of abortions in 23 animals in the third gestation in this laboratory, compared with those in 19 animals in the fourth or in 10 in the fifth gestation in the experiments at the Bureau are significantly different. At the present writing, the factor or factors responsible for the considerable difference in results are unknown. More tests with larger numbers, if possible, are required to provide an answer to this important question of the duration of the protection afforded by calfhood vaccination.

Individual Response to Infection.—Most workers in brucellosis would agree that there is considerable difference among normal individuals in their response to the same dosage of *Br. abortus*. If, for example, 1,000 animals could be assembled at random, and be exposed in about the same stage of pregnancy to a reasonable but adequate dose of virulent *Br. abortus*, from 100 to 300 individuals of the 1,000 would be expected to produce normal calves, most of the others would abort, and some would be intermediate in reaction (*i. e.*, produce living but weak calves, or dead calves at about full term). It is proposed that, aside from errors of technique, the physiologic mechanisms governing the response of each animal—whether susceptible, intermediate, or resistant—would be determined largely by its inheritance. This proposal is made without experimental evidence in brucellosis of cattle beyond a few observations that "resistance appeared to run in a family." Such observations are no more than suggestive of the possible role of heredity. However, analogy with the proved factor of heredity in making for resistance and susceptibility to various infections with microörganisms in plants and in animal species other than cattle makes such a proposal almost a certainty.

On the premise that heredity is a factor in determining the response of an individual to brucellosis, it is further proposed that the protection afforded by vaccination is also the result of the interaction between a genetically variable host and a genetically variable pathogen. Within a normal population of random mated animals, at one extreme there would be a few of great susceptibility, at the other extreme a few of high resistance, and those in between would have varying degrees of susceptibility. That is, between the two extremes (highly susceptible animals on one end and at least reasonably resistant ones at the other) would be found the greatest proportion of the total. A curve depicting the distribution of these various grades of susceptibility would be a probability curve, with the average proportion of susceptibles as the mean. Following vaccination, those which genetically are the most susceptible would be the group least likely to be protected against a virulent organism, and for the shortest time; the greater proportion of susceptibles, other than those not in the previous class, would be protected for varying periods; those nearest the threshold of resistance would be protected most definitely and probably for a longer period; and those naturally resistant would not need the protection of acquired immunity through vaccination, but their resistance would be increased by the stimulus of the vaccination. The individuals innately most susceptible would have the shortest duration of whatever resistance to the organism they had acquired. The less the susceptibility, up to the threshold of resistance, the greater the acquired immunity and the longer would be its duration. However, since the resistant individuals cannot be recognized at present, except by their reaction to an infection, vaccination must be done on all animals.

This concept cannot be supported by experimental work with *Br. abortus* in cattle. The only evidence along this line known to the authors is that by Gowen[16, 17] who showed that in six inbred lines of mice, varying genetically in response to a lethal does of *Salmonella typhimurium*, the response to a heavy infecting dose after vaccination with heat-killed organisms paralleled the genetic resistance of these strains. That is, all the animals were protected to some extent by the vaccination, but the innately resistant were protected much more than the innately susceptible. In essence, the degree of protection depended upon the degree of innate resistance.

An alternative hypothesis is that the immunity engendered by vaccination with strain 19 is an infection immunity analogous to the infection immunity of tuberculosis and depending upon the persistence of the living immunizing organism with the host. Should this be the case, persistence of the vaccine strain of the organism would be expected to be greatest in the innately susceptible animals, with attendant resistance to reinfection. Under either proposal, however, it is clear that the level of resistance engendered by vaccination is directly related to the innate resistance of the individual animal.

SUMMARY

A summary of the results of exposing normal animals by way of the conjunctiva to at least a million organisms—usually many times this number—of *Brucella abortus* showed that in 36 trials there had been 303 abortions among a total of 353 animals. The variations of the percentages of abortions around the average (85.84%) would occur infrequently by chance alone, meaning that the underlying probability (85.84) of abortion could not be considered uniform from trial to trial.

In contrast, various tests of the protection during the first gestation period afforded by calfhood vaccination gave reactions which appeared to be homogeneous from test to test. Quite differing results, however, were obtained in different laboratories from challenging during later pregnancies the immunity acquired by calfhood vaccination.

A review of the effects of varying dosages of the organism reveals no definite correlation between the number of organisms and the proportion of abortions.

The variation by virtue of random sampling in the number of abortions to be expected in tests using small numbers and with different probabilities of abortions is discussed.

All things considered, it is highly desirable for agreement to be reached among workers in experimental brucellosis as to an adequate but not overwhelming dose of the causative organism, the route of exposure, and the stage in gestation at which exposure is made in experiments in cattle. The results from different laboratories would then be much more comparable than they are at present. Also, the causes of the variations in the results from different laboratories could be more easily determined, other than those which are to be expected on the basis of random sampling.

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Studies on Johnin

VI. The Relationship of the Allergens of *Mycobacterium Paratuberculosis, Mycobacterium Tuberculosis* var. *Avium, Bovis,* and *Hominis,* and *Mycobacterium Phlei*

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MEMBERS OF THE group of organisms classified by Bergey[1] under the genus Mycobacterium have occupied, and still are occupying, a place of great interest in the fields of both veterinary and human medicine. In veterinary medicine, paratuberculosis, or Johne's disease, is a widespread and increasingly important disease of cattle in the United States. Although the tuberculosis eradication program, which was inaugurated by the Bureau of Animal Industry in 1917, has given the United States the lowest incidence of bovine tuberculosis of any nation in the world, it still leaves this country with the disease within its boundaries. The avian species of this genus is widespread in some areas and causes considerable loss in both chickens and swine. "No visible lesion" tuberculin reactors are of greater and greater concern because of the increase in this group of animals in relation to the number of lesion cases found and because of the lack of complete understanding of their cause. This objective survey indicates the desirability for studies which might enlarge our knowledge of the antigenic relationships within the genus Mycobacterium and thus point the way for possible improvement in the specificity and standardization of Mycobacterium allergens. This, in turn, would make it possible to obtain sooner our goal of living without tuberculosis and Johne's diseases of cattle.

In a previous paper,[2] it was reported that cattle sensitized with *Mycobacterium paratuberculosis* could be used for the comparative testing of many johnins. It seemed likely that additional information regarding the antigenic relationship between members of the genus Mycobacterium could be obtained if cattle were sensitized by one or another of the species and simultaneously tested with each of several allergens prepared from synthetic culture mediums on which members of this genus had grown. Therefore, a study was made of the relationship of the potency of different allergens when tested on groups of cattle that had been sensitized with acid-fast organisms of different species.

This work was conducted at the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, Auburn, Alabama.

MATERIALS AND METHODS

Fifty cattle, divided into five equal groups, were used. Each group was sensitized with one of the following organisms: *M. paratuberculosis, Mycobacterium tuberculosis* var. *bovis, Mycobacterium tuberculosis* var. *hominis, Mycobacterium tuberculosis* var. *avium,* and *Mycobacterium phlei.* The allergens used were seven johnins, one phlein, two avian tuberculins, two *hominis* tuberculins, and one *bovis* tuberculin. Each allergen was tested four times in three dilutions on each animal, making a total of 156 injections on each animal, or a total of 7,800 injections on the fifty animals.

Fifty grade Jerseys, 2 or more years old, weighing from 450 to 750 lb., and negative to both johnin and tuberculin, were purchased for these experiments. Ten animals were placed in each of five pens and fed a good grade of alfalfa hay and mixed cattle feed. One month was allowed for the animals to adjust themselves to the new surroundings and feed before sensitizing was started.

The organisms used for sensitizing the cattle were all grown on Dorset and Henley's[3] synthetic medium, except *M. tuberculosis* var. *bovis,* which was grown on glycerol broth medium. Each animal was injected intraperitoneally with 60 cc. of a mineral oil suspension, which contained 3 Gm. of heat-killed, moist organisms. The oil suspensions were prepared by the method described in an earlier publication.[4]

All tuberculins were prepared by the method described by Dorset and Henley.[3] The seed

Medium Formula

l-asparagine	14.0 Gm.
Dipotassium phosphate	1.8 Gm.
Sodium citrate	0.9 Gm.
Magnesium sulphate	1.5 Gm.
Ferric citrate	0.3 Gm.
Dextrose	10.0 Gm.
Glycerine	100.0 Gm.
Water to make	1000.0 Gm.

culture is inoculated on a sterile synthetic medium. The newly inoculated medium is then incubated from ten to twelve weeks at 37.5 C. The cultures are then sterilized and filtered. The filtrate, after addition of a small amount of glycerol, is evaporated to one fifth its original volume, and an equal amount of 1 per cent phenol solution is added to bring the total volume to 40 per cent of the original amount, which affords an O. T. concentration equivalent to 25 per cent. Therefore, they are considered as 1 : 4 dilutions. The johnins were prepared in a similar manner, and the original dilution of these is also considered as 1 : 4.

All sensitivity tests reported in this paper were carried out with 0.2-cc. injections administered intradermically with either ¼ or ⅜ in. 27-gage needles, between the fourth and sixteenth weeks following the sensitizing inoculations. A 1-cc. glass syringe, graduated in 1/100 cc. and in minims, and having a blue plunger, was employed for all injections.

Skin thickness readings at each point of injection were made and recorded prior to injection of the allergen and forty-eight hours after injection. All readings were made with a dermal thickness gage[6] with readings recorded to ¼ mm. The initial readings were then subtracted from the 48-hour readings and the number of millimeters increase resulting from each injection was thus obtained. Four tests were carried out on each animal at monthly intervals. The four readings of each material were averaged to arrive at the figures reported on the graphs.

The following illustrates the method used for computing the number of potency units present in an allergic material.

Calculation of Potency Units

a Dilution tested one O.T. diluted	b Thickness developed in mm.	Potency units (a x b)
to 4	10	40
to 40	5	200
to 400	1	400
Total		640

This allergen would have 640 potency units.

RESULTS

As shown in the accompanying graphs, differences in the amount of allergen in the seven lots of johnin were readily seen when these materials were injected intradermically into homologously and heterologously sensitized cattle. Three of the seven johnins tested (1, 2, and 3) were found to be highly potent; two (4 and 5) were moderately potent; and two (6 and 7) were of limited potency. This is particularly noticeable in the groups sensitized with *M. tuberculosis* var. *avium* and *M. paratuberculosis*. This relationship showed a remarkable uniformity on the four different tests made at monthly intervals, even though the sensitivity of the animals gradually decreased.

Two *avium*, one *bovis*, and two *hominis* tuberculins were moderate in potency when tested simultaneously on the same *M. paratuberculosis*-sensitized cattle. Phlein showed very little potency on all sensitizations tested except the *M. phlei*-sensitized animals. The johnins and tuberculins showed such a small amount of active material when tested on *phlei*-sensitized animals that the readings could not be recorded on a graph similar to the ones shown here.

The two avian tuberculins which were titrated in this experiment were of nearly equal potency. Two highly potent johnins (1 and 2) were found to be nearly equal in potency to the two avian tuberculins when tested on *M. tuberculosis* var. *avium*-sensitized cattle. The two *hominis* and the one *bovis* tuberculins tested in this experiment contained very little potency when tested on the *M. tuberculosis* var. *avium*-sensitized cattle. The potency was less than half the amount that resulted when these tuberculins were tested on *M. paratuberculosis*-sensitized cattle.

One *bovis* tuberculin was titrated in this experiment. The potency manifested by it on *M. tuberculosis* var. *bovis*-sensitized animals was approximately equal to the potency shown to be present in johnin (3), when this johnin was tested on *M. paratuberculosis*-sensitized cattle.

The potencies manifested by the two most potent johnins (3 and 2) when tested on *M paratuberculosis*-sensitized cattle, were about one fourth and one fifth, respectively, of the potency demonstrated to be present in the *bovis* tuberculin when both types were tested on *bovis*-sensitized cattle. The two avian tuberculins showed less potency for *bovis*-sensitized cattle than was demonstrated to be present in the most potent johnins. The two *hominis* tuberculins contained a larger amount of active material for the *bovis*-sensitized cattle than the *bovis* tuberculin, when tested simultaneously on these cattle.

The two *hominis* tuberculins were found to be nearly equal in potency on all groups of cattle tested. The *bovis* tuberculin contained a comparatively large amount of active material for *hominis*-sensitized cattle. All seven johnins and both avian tuberculins were found to contain active material for *hominis*-sensitized cattle, but only in limited amounts (*see* graph).

A state of extreme sensitivity was produced in cattle by the intraperitoneal injection of *M. phlei* suspended in mineral oil. However, this sensitivity lasted only about one month and then decreased rapidly. The loss of sensitivity was much slower in the animals sensitized with *M. tuberculosis* var. *bovis*, *M. tuberculosis* var. *hominis*, *M. tuberculosis* var. *avium*, *and M. paratuberculosis*. The average results of the readings in potency units on the *phlei*-sensitized animals were as follows: johnins, 4; *hominis* tuberculins, 5.4; *bovis* tuberculins, 6.2; avian tuberculins, 2.6; and phlein 2,265.0 on the first test, gradually decreasing to 317.0 on the fourth test, sixteen weeks after sensitization.

This experiment demonstrated that there was an almost complete lack of heterogenetic antigen in the *M. phlei* cultures used to sensitize these animals. This evidence indicates that *M. phlei* would play only a very minor rôle in causing reactions in animals which were being tested with either johnin or one of the tuberculins.

SUMMARY AND CONCLUSIONS

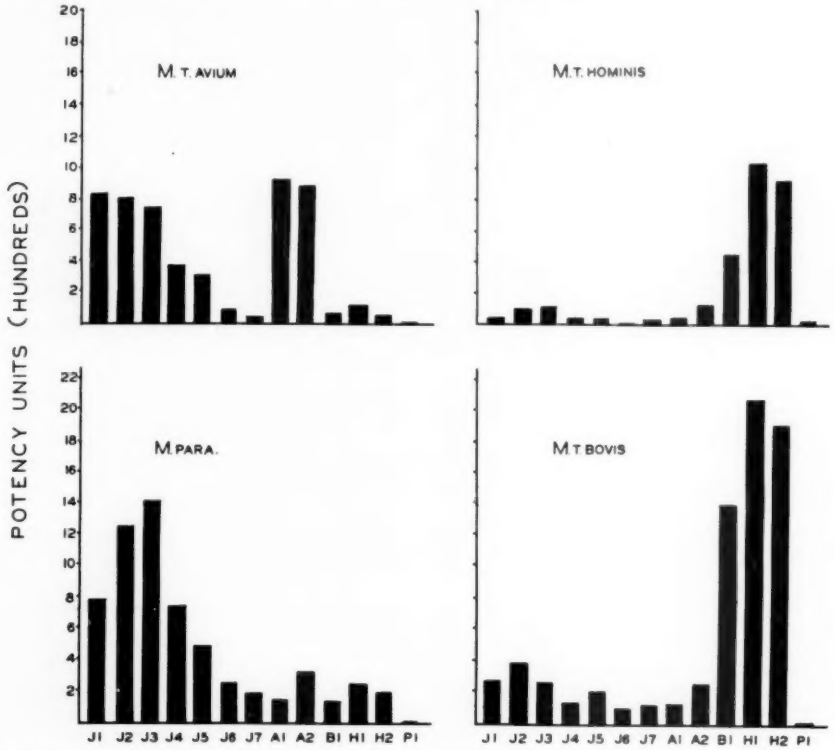
A method is described for experimental comparison and evaluation of Mycobacterium allergens in cattle sensitized with different species of Mycobacterium. Based upon this method, the following comparisons and evaluations were made:

1) The seven lots of johnin tested had a wide range in potency.

2) When tested on *avium*-sensitized cattle, the two most potent johnins were found equal in potency to the two avian tuberculins used in the tests.

3) The most potent johnin contained more nonspecific allergens for *bovis*-sensitized cattle than were contained in any of the avian tuberculins tested.

4) Avian tuberculin and johnin contained some nonspecific allergens for *hominis*-sensitized cattle.



J = johnin; A = avian tuberculin; B = *bovis* tuberculin; H = *hominis* tuberculin; P = phlein.

Fig. 1—Comparative potency of different allergens on cattle sensitized with different species of Mycobacterium.

5) Both *bovis* and *hominis* tuberculins contained more nonspecific allergens for *Mycobacterium paratuberculosis*-sensitized cattle than for *avium*-sensitized cattle.

6) The two *hominis* tuberculins were in relatively close agreement, as were also the two avian tuberculins, in all groups of cattle.

7) *Mycobacterium tuberculosis* var. *bovis*, *Mycobacterium tuberculosis* var. *hominis*, *Mycobacterium tuberculosis* var. *avium*, and *M. paratuberculosis*-sensitized animals reacted to a potent allergen prepared from any one of these organisms.

8) *Mycobacterium paratuberculosis* and *M. tuberculosis* var. *avium* sensitizations are possible factors to be considered in "no visible lesion" reactors to the tuberculin test.

9) *Mycobacterium phlei* sensitivity is of minor importance in causing nonspecific reactions to either johnin or tuberculin.

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Histologic Changes in Explanted Bovine Epithelial Tongue Tissue Infected with the Virus of Foot-and-Mouth Disease

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THIS IS a report on the histopathologic alterations occurring in explants of bovine lingual mucous membrane cultured in medium containing foot-and-mouth disease virus. The purpose of the investigation was to determine whether any lesions would be found that could be attributed to the action of the virus.

On histologic study of vesicles produced in the course of natural or artificial infection of animals, one cannot be certain which part of the lesion is due to the action of the virus and which part is due to the reaction property of the infected animal. Circulatory changes play an important rôle in the formation of the vesicle of natural and artificial infection. There is an inflammatory reaction with abundant exudation, in the course of which large, fibrinous formations are often observed at the bottom of the vesicles.

Little is definitely known about the earliest stages of the vesicle formation. According to the investigations of Galloway and Nicolau carried out on guinea pigs, ferrets, and rabbits, the first cells of the stratum spinosum attacked by the virus become loosened from the others, forming foci of isolated cells having a stronger eosinophilic-staining reaction than the rest. This represents the stage of the primitive vesicle. There follows an inflammatory reaction which is at the first serous and afterwards of a more exudative, cellular character. In the early stages of the vesicle formation, it is remarkable that the stratum germinativum does not become involved by the pathologic process. Apparently, these cells remain intact. It is not known whether these cells participate in the multiplication of the virus, or merely let the virus pass. It must be admitted that they have little or no affinity for the virus of foot-and-mouth disease, whereas the cells of the stratum spinosum are obviously of such chemical nature that the virus can multiply in them.

Because of the vascular reaction, the vesicle is formed. It may be supposed that the fluid exudate, which shows considerable resemblance to blood serum, diffuses through the deep cell layers of the skin or mucous membrane and accumulates in the intercellular spaces of the stratum spinosum which have been accentuated by the shrinking of the cells.

Since an inflammatory reaction does not occur in the tissue explants infected with the virus, any changes occurring in the cells may be attributed mainly to the action of the virus. A study of the lesions in tissue explants is therefore considered important for a better understanding of vesicle formation in the living animal and for the elucidation of the earliest pathologic changes.

MATERIALS AND METHODS

The tissue used was taken from the deep layers of lingual mucosa of cattle that had never been affected with foot-and-mouth disease. These deep layers included the stratum germinativum, the stratum spinosum, and part of the underlying connective tissue. The inclusion of muscular tissue was avoided because its presence soon renders the explants acid and damages the virus.

The superficial layer of the mucosa was removed in such a way as to keep the underlying epithelium sterile. This was done by splitting the mucosa at the tip of the tongue and then rolling down the superficial part of the mucous membrane with the aid of a metal clamp. With this technique, separation or splitting of the superficial layer from the deep layers takes place in the upper part of the stratum spinosum. The epithelial tissue that is most susceptible to the virus remains on the tongue. The deep epithelial tissue was removed with sterile safety razor blades and put into Tyrode's solution. This tissue was minced with sterile double-curved scissors in double Petri dishes to prevent contamination from the air as much as possible. To the minced material were added buffer solution (primary and secondary sodium phosphate), foot-and-mouth disease virus, and 5 drops of penicillin 1: 5,000, and streptomycin 1: 50,000 solutions. The mixture was allowed to stand overnight in a refrigerator to permit the virus to have prolonged contact with the cells and thus favor its adsorption to, or penetration into, the cells.

The virus-tissue suspension was then added to Baker's nutrient medium. A simple type of cultivation flask was adequate. Common, wide-necked Erlenmeyer flasks were suitable for the purpose. The nutrient medium had to be aërated to provide the cells with a sufficient

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quantity of oxygen because the fluid column frequently was as high as 5 to 8 cm. The aëration was done with carbogen, a gas mixture consisting of 95 per cent O_2 and 5 per cent CO_2. The gas supply was regulated with a reduction valve mounted on a gas flask and with the aid of a set of fine regulating valves. The gas was fed in by a glass pipette ending in a fine point and provided with a sterility protector consisting of a glass bulb filled with cotton. A second tube was provided for evacuation of the superfluous gas. As a further precaution to ensure sterility, the fissure between the stopper and the flask was filled with a rubber-vaseline mixture.

In consequence of the aëration, a foam formed above the fluid. In order to avoid microbial contamination, foaming was controlled by appropriate regulation of the aëration apparatus. The flasks thus prepared were put into an incubator at 37 C. for twenty-four hours. The fine regulating part of the aëration apparatus was also kept in the incubator to heat the gas in advance.

After incubation, the explants were fixed in Bouin-Brazil fluid and embedded in paraffin. Sections were stained with hematoxylin and eosin. Best's stain was used to examine for glycogen, sudan III for fat, and phloxine-water blue for inclusion bodies.

In addition to examination of explants incubated for twenty-four hours, a number of explants were studied after four, eight, ten, twelve, fourteen, and sixteen hours of incubation. Examination of explants infected with A type virus was repeated ten times in succession, and similar studies of explants infected with O type virus were made twice. On each occasion, a parallel cultivation without the virus was carried out with tissue from the same tongue to serve as a control and thus exclude any doubt as to the specificity of the histologic changes. As the pathologic alterations observed in all cases were similar, a general description of the findings will be sufficient.

In sections of the explants that had been incubated in Baker's fluid without virus, there was no alteration in the continuity of the structure of the mucous membrane. The various layers of epithelial cells formed one continuous tissue. There was a distinct difference between the cells of the stratum germinativum and those of the stratum spinosum. The chromatin content of the nuclei was considerably larger in the cells of the stratum germinativum.

The histopathologic alterations found in comparable explants containing virus were reminiscent of the natural vesicle as observed in cases of foot-and-mouth disease. The cytoplasm of the stratum spinosum cells was swollen and showed an accentuated eosinophilic-staining reaction. The nuclei were pyknotic and fragmented or showed peripheral displacement of the chromatin. However, the most striking change was a loss of the interrelationship of the cells, exactly as is to be seen on histologic examination of the vesicle in a diseased animal. The cells appeared to be separated one from another. Sometimes, the original cell limits were still visible as a fine network of protoplasm. It appeared as though lysis of the protoplasm had occurred, beginning at the periphery of the cells and causing separation of the originally adhering cells. After this, degenerative changes of the nuclei became evident, consisting of peripheral displacement of the chromatin, pyknosis, and rhexis. Isolated cells with pyknotic nuclei re-

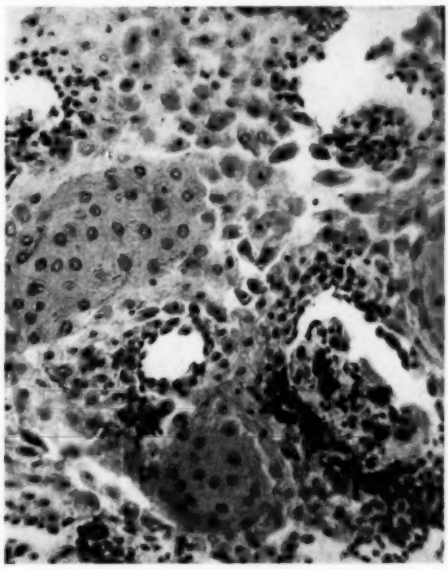

Fig. 1—Showing a tangential section through the explanted mucous membrane of the bovine tongue infected with foot-and-mouth disease virus and incubated for twenty-four hours. Cross section through papular bodies surrounded by isolated degenerating cells of the stratum spinosum. Solid masses of hornified epithelium.

sembled the red blood corpuscles of birds. Shrinking of the nuclei was observed to the extent that they seemed to lie in a vacuole. Frequently, the entire cell was pyknotic.

It appeared as though a proteolytic ferment were present, destroying the coherence of the cells and causing them to disappear partially or totally. Here and there a confluence of the protoplasm of a number of cells and a grouping of their nuclei resulted in formations re-

sembling giant cells. The over-all picture was one of colliquative necrobiosis, mainly affecting the cell layers close to the stratum germinativum.

This histologic picture resembles very much the vesicle of the animal suffering from foot-and-mouth disease with the exceptions, however, that no lymph was formed and leucocytes were not present. In the case of infected explants, the lesions may be considered as vesicles in which the rôle of the lymph is played

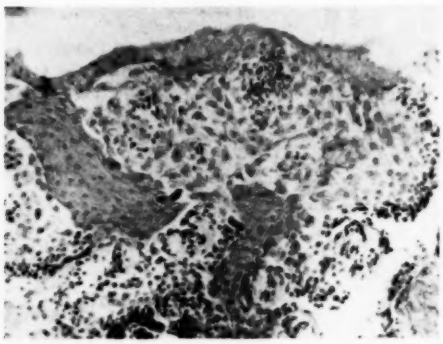

Fig. 2—Vesicle formation in infected explantations of the mucous membrane of the bovine tongue. Parts of the normal skin are still visible. The isolated epithelial cells of the stratum spinosum still adhere by a fine protoplasmic network.

by the nutrient medium and fluid given from the degenerated cells.

In many spots, the changes in an area showing the effects of the virus were so pronounced that the entire cell mass above the area was elevated as can always be observed in the natural infection. In these instances, there also was definite degeneration of the cells of the stratum germinativum indicated by shrinking of the protoplasm and the nuclei. The degenerated cells stained strongly with hematoxylin. Sometimes, separation of the cells also was present to a considerable extent in the epithelial tissue under the stratum corneum.

Sections stained with sudan III and sections stained by Best's method failed to show either fat or abnormal quantities of glycogen in the cells. Sections stained with phloxine did not show any inclusion bodies that could be considered as typical, since bodies of the type observed were also present in tissue explants that did not contain the virus.

Explants infected with virus and incubated four hours did not show any alterations that could be ascribed to the action of the virus. After eight hours' incubation, slight pathologic alterations were found on careful examination. Tangential sections of the mucosa at a depth where the papular bodies are seen showed beginning separation and a more eosinophilic-staining reaction of a small number of cells. After ten, twelve, and fourteen hours, the cellular alterations and separation of the cells were more distinct. After sixteen hours' incubation, the pathologic picture was nearly as well developed as after twenty-four hours.

DISCUSSION

These findings show that the virus of foot-and-mouth disease is capable of causing pathologic changes in explanted tissue from the mucosa of bovine tongues. In many respects, these changes resemble the cell alterations and the vesicle formation in diseased animals. Since the action of the vascular system is eliminated in tissue explants, the lesions found may be attributed to the action of the virus. It is a remarkable fact

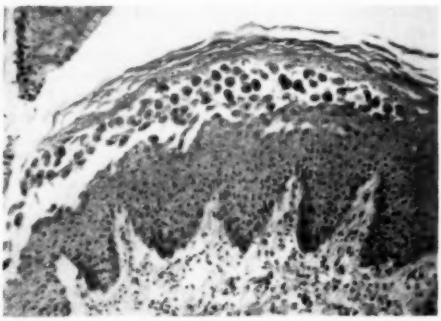

Fig. 3—Showing vesicle formation in the superficial part of the stratum spinosum of explanted, infected bovine tongue tissue.

that these lesions resemble to such an extent the vesicle formation in the diseased animal. These findings are important to an understanding of vesicle formation in general and particularly to such lesions in foot-and-mouth disease. It seems obvious that vesicles originate in a degeneration of the epithelial cells that causes the cells to shrink and the intercellular connections to disintegrate. The space between the degenerating cells becomes filled with fluid. In spontaneous vesicles, this fluid consists mainly of blood serum, as shown by analysis of the vesicle lymph, whereas in mucosal explants, this fluid is derived from the nutrient medium. Since the explanted tissue fragments were very small, the formation of gross vesicles has not been observed to the present time.

Like the formation of a natural vesicle, most of the vesicle formation in tissue cul-

tures took place in the deeper zones of the stratum spinosum, although cell alterations were sometimes seen in parts of the mucosa closer to the stratum corneum. The stratum germinativum mostly remained unchanged, although the cells of this layer sometimes showed degeneration indicated by pyknosis of the nuclei. The quick healing of the vesicles of foot-and-mouth disease may be attributed to the failure of the cells of the stratum germinativum to become involved.

The fact that the virus of foot-and-mouth disease produces histologically recognizable changes in explanted lingual mucosa of cattle susceptible to the malady might be of great importance in another way. It is still desirable to develop a laboratory method for differentiation between the virus types so that the use of cattle for that purpose would not be necessary. The method of complement fixation has not always been satisfactory, according to the experience of many laboratories. It might be possible to differentiate the virus types with the aid of the pathologic changes occurring in tissue culture.

Under present conditions, the exact typing of a virus strain from the field requires a number of cattle. Typing of guinea pigs is not like typing of cattle. A method for typing virus samples with tissues from susceptible cattle would therefore be of considerable value. The following procedure is proposed for typing foot-and-mouth disease virus in tissue culture: First, one must have strong, pure immune serums against the O, the A, and the C types of virus. It would be advisable to use cattle for preparation of immune serums and to inject the animals several times. Explants of lingual mucosa from the tongues of fully susceptible cattle are placed in different flasks of nutrient medium containing, respectively, the O, A, and C type immune serum instead of normal serum as prescribed in Baker's fluid. The virus to be typed is added to the flasks. Failure of explants to show lesions after twenty-four hours' incubation would indicate that the unknown virus corresponded to the virus used in the preparation of the immune serum in the flask. As a control, some explants should be incubated with nutrient medium containing normal serum. Work on this method is being done at present and a report will be made as soon as definite results are available.

SUMMARY

A description is given of the histologic changes occurring in the deep explants of bovine lingual mucosa infected with foot-and-mouth disease virus. The explants studied were obtained from cattle that had never been affected with the disease. The tissue changes observed resemble, in many respects, the vesicles occurring in diseased animals. The type of degeneration resulting from the action of the virus is colliquative necrobiosis.

The cellular changes were seen mostly in the stratum spinosum near the stratum germinativum, but sometimes more in the neighborhood of the stratum granulosum. The stratum germinativum is rarely involved.

The possible use of this culture phenomenon for the differentiation of the virus types is discussed.

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Survey of Animals for Inapparent Infection with the Virus of Japanese B Encephalitis

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THE PURPOSE of this paper is to report on the compiled data of neutralization and complement fixation tests for Japanese B (Nakayama) virus which were carried out periodically during the summer months of 1947 on normal domestic animals of various species in the enzoötic areas of Japan. These data give information concerning the extent of virus dissemination among domestic animals during the epizoötic season and will aid in the natural history discussion of the virus and mode of infection of this disease.

Complement fixation tests were used extensively during the epizoötic of equine encephalitis in Japan in the summer and autumn of 1947.* The data reported herein will supply some of the necessary information for analyses of the serology of this equine malady.

MATERIALS AND METHODS

Animals.—Horses, cattle, sheep, dogs, cats, and rabbits were used in this study.

Fifteen draft horses, which were born in Hokkaido (an area generally considered nonepizoötic and nonepidemic) during the years 1944 to 1946, were imported to Okayama Prefecture in 1946 and 1947 (the last one in July, 1947), and distributed throughout rural areas east of Okayama City, which is reputedly one of the highly endemic areas for Japanese B encephalitis.

Ten cows, which were born in Hokkaido during 1940 to 1945, were imported into the same areas as the horses during December, 1946.

Eight sheep, born on a farm in Okayama Prefecture during the years 1943 to 1945 and raised there until the time of this experiment, were studied.

Another group of 8 Japanese horses of unknown age and history were stabled in Yokohama and were used as a part of this group survey.

During the early part of August, 1947, 8 dogs, 10 cats, and 10 rabbits of unknown ages, purchased in Hokkaido and transported to Kasukabe, a rural town in Saitama Prefecture approximately 40 miles north of Tokyo, constituted another group of animals for this survey.

Another group of 9 rabbits was purchased locally from Kasukabe and raised in hutches in urban Tokyo. These rabbits were known to be negative for neutralizing antibodies against Japanese B virus, based upon previous neutralization tests.

Serums.—The blood specimens from Okayama, Yokohama, and Saitama were sent refrigerated as whole blood or serums to the Tokyo laboratory. The serums were separated immediately and stored in a blood bank refrigerator (2 C.) without any preservative until tests were completed.

Neutralization Tests.—The neutralization tests were carried out with Nakayama strain of Japanese B virus, which was stored as a centrifuged 20 per cent suspension of infected mouse brain in 10 per cent heated (at 56 C. for 30 minutes) rabbit serum saline in an insulated box containing solid CO_2. Two-tenths cubic centimeter of unheated, undiluted serums were added to equal amounts of 3 dilutions of the virus (10^{-5}, 10^{-6}, and 10^{-7} in final dilutions). The mixtures of the serum and virus were heated in a water bath at 37 C. for two hours and then inoculated intracerebrally into 4-week-old mice. Four mice were used per dilution of the virus. The mice were observed for two weeks. Undiluted, heated rabbit serum known to be negative for neutralizing antibodies against Japanese B virus was used as a control in each individual test. The neutralization index, representing the ratio between the LD50 of the unknown serums and the control, was calculated in the usual manner.

Complement Fixation Tests.—The tests proper were carried out according to procedure outlined by Casals and Palacios[1] with overnight incubation of the serum-antigen-complement mixtures, and complement titrations in the presence of antigens in an electric refrigerator at 5 C. Usually twofold serial dilutions of serum starting from 1:2 were utilized, but in some instances a 1:4 mixture was the initial point of dilution.

Japanese B virus (Nakayama strain) and a control of normal mouse brain were used in all tests. In addition, as indicated, the antigen of Western equine encephalomyelitis virus was utilized. These antigens were prepared from infected mouse brains. Ten per cent suspensions in physiologic saline containing 2 per cent normal heated guinea-pig serum were centrifuged horizontally at 2,000 r.p.m. for thirty minutes after overnight incubation in the refrigerator. The supernatants, without freezing and thawing, were then centrifuged at 15,000

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*The results of our investigations of this equine epizoötic will be reported in a separate paper.

r.p.m. for one hour on the angle head of an International centrifuge under refrigeration with solid CO_2. To the clear supernatant, constituting the antigens, was added merthiolate to 1: 10,000 final concentration and stored in a refrigerator at 5 C.

The serums used in the above tests were inactivated as follows: equine, bovine, feline, and ovine serums 60 C. for twenty minutes, rabbit and canine serums 62 to 63 C. for forty minutes, or 65 C. for twenty minutes. The latter two species of animal serums were often anticomplementary even though the higher temperature was used for inactivation.

RESULTS

Results of the neutralization and complement fixation tests are summarized in table 1. Because of variations in animal supply, it was not possible to conduct all neutralization tests at the same time, nor was exactly the same titer of stock virus available for each of the runs. In later work, this has been possible and somewhat less variation has been evident.

The usual figures have been used in applying descriptive terms to the neutralization indexes, *i.e.*, an index of 50 or greater has been termed positive, 10 to 49 termed equivocal, and less than 10 called negative. A fourfold increase in complement fixation titers has been considered significant.

On the initial serum neutralization tests of Okayama horses, drawn on July 21, 1 and 4 of 15 horses were positive (neutralization index 50 or more) and equivocal (neutralization index between 10 and 49), respectively; the remaining 10 horses portrayed a negative neutralization index. All the negative and equivocal animals became positive on subsequent bleedings during August or September. Complement fixation tests on these horses, which were negative on earlier specimens, became specifically positive in agreement with the results of neutralization tests. One horse which had a positive neutralization index on its initial serum specimen remained positive during subsequent neutralization tests on later specimens. However, complement fixation tests of this animal portrayed negative results on the initial specimen and became specifically positive on the second and later serum samples.

Neutralization tests conducted upon Okayama equine specimens on Dec. 23, 1947, and Jan. 12, 1948, revealed that all the horses had a positive neutralization index. In contrast, complement fixation tests showed that positive reactions were either considerably reduced in titer or negative in all cases.

Four of 8 horses in Yokohama remained negative for neutralizing and complement-fixing antibodies during the period of this experiment. Horse 30 (Miyasawa) had an initial neutralization index of 10 on the serum drawn July 29, while subsequent specimens had indexes of 50 or more with complement fixation titers remaining negative throughout the survey. Two other horses (31 and 32) had a positive neutralization index from the beginning and remained so, while companion complement fixation tests were negative for all specimens. Horse 17 (Weaver) had a neutralization index of less than 10 for the initial serum specimen, afterwards exhibiting a definitely positive index. Although complement fixation tests were not conducted on the first two specimens, subsequent bleedings showed an extremely weak response against Japanese B antigens.

Among Okayama cattle, 1 was initially equivocal and 4 were positive; the remaining 5 animals had a negative neutralization index. Four of these 5 negative animals became positive later with the equivocal one exhibiting a positive index for the serum of September 19. All of the initially positive animals remained so throughout sub-

TABLE 1—Summary of Neutralization and Complement Fixation Tests

Species	Locality	No. of animals	Neutralization Tests*			Complement Fixation Tests†	
			Pos. to pos.	Neg. or equivocal to positive	Neg. to neg.	Neg. to pos.	Neg. to neg.
Horses	Okayama	15	1	14	0	15	0
Horses	Yokohama	8	2	2(?)	4	1(?)	5
Cattle	Okayama	10	4	5	1	1	9
Sheep	Okayama	8	0	1	7	0	8
Dogs	Saitama	8	0	0	8	0	8
Cats	Saitama	10	0	0	10	0	10
Rabbits	Saitama	10	0	0	10	0	10
Rabbits	Tokyo	9	0	0	9	0	9
TOTAL		78	7	22	49	17	59

*Neutralization tests: Positive = neutralization index 50 or greater. Equivocal = neutralization index 10 to 49. Negative = neutralization index less than 10.
†Complement fixation tests: Positive = fourfold or greater increase in titer over that of normal mouse brain or Western equine antigens.

sequent bleedings, the one exception being the second specimen of horse 8 (Shinei), which had a neutralization index of 10 on the second bleeding. Many nonspecific reactions were observed in complement fixation tests of Okayama cattle. Results of these tests, particularly those of animal 9 (Carnation Romeo), were noteworthy because of negative titers of the first three specimens, while the last serum specimen, taken on September 19, portrayed a fourfold increase in complement-fixing titer against Japanese B antigen, as compared to Western equine encephalomyelitis and normal mouse brain antigens. In addition, this animal became positive for neutralizing antibodies from the second specimen in August.

All neutralization tests conducted on Okayama sheep serums were negative except for sheep 4, which was negative on initial bleeding, becoming positive with the specimen of September 9. Complement fixation tests were all negative. Dogs, cats, and rabbits were all negative for both neutralizing and complement-fixing antibodies, remaining so throughout the period of this observation.

An extensive inapparent infection is clearly demonstrated among horses and cattle in Okayama. Thus, 1 of 14 horses without neutralizing antibodies in July obtained a positive neutralization index on the serum specimen of August 13. The remaining 13 horses neutralized Japanese B virus during tests conducted on serum samples during September. On the other hand, only 2 of the 6 horses in Yokohama which were initially negative changed to positive in July and August, respectively. Neither of these animals showed the clear-cut changes evident in the Okayama horses. It seems probable that horses 17 and 30 should be considered as having had previous experience with the virus, since the original indexes were borderline, and neither animal showed a significant complement fixation response. The results are otherwise unexplainable.

Six cases of equine encephalitides-type infections were reported in Okayama Prefecture during the summer of 1947, while none were reported in Kanagawa Prefecture, within which the City of Yokohama is located. Laboratory studies confirmed 4 clinical cases of Japanese B encephalitis in human beings in Okayama Prefecture, while none were reported in the Tokyo or Yokohama area and Saitama Prefecture.

In contrast, only 1 of 8 sheep with initial negative neutralization indexes became positive late in September. These animals were quartered on a farm remote from the area where the horses and cattle were located. It is not known whether this poor dissemination of the virus among sheep, in comparison with the extensive one among horses and cattle, is due to difference of locality or to one of family species, as shown by Sabin, Ginder, and Matumoto,[2] *i.e.*, between human beings and domestic animals in higher endemic areas in Japan during the summer of 1946.

There was no, or insignificant, dissemination of the virus among dogs, cats, and rabbits at Kasukabe, in Saitama Prefecture, nor among rabbits in Tokyo during the duration of this experiment. Thus, all the animals remained negative for neutralizing antibodies during the summer of 1947.

No complement-fixing antibodies were found in serums which did not have neutralizing antibodies.

Complement-fixing antibodies may be produced without evidence of infection with Japanese B virus in horses. Thus, 14 horses in Okayama, which produced neutralizing antibodies of significant titer in August or September, were highly positive and specific for Japanese B virus in complement fixation tests conducted on the companion serums bled during the same months.

Serum specimens obtained on Dec. 23, 1947, and Jan. 12, 1948, contained either no complement-fixing antibodies or only very low titers. These data suggest that a positive complement fixation test for Japanese B virus in horses means a recent infection by the virus.

One Okayama horse is of interest. This animal had a positive neutralization index on the initial serum specimen of July 21, while the companion complement fixation test was negative but became positive in the second serum of August 13. There are two possibilities: (a) Positive neutralization on the initial serum was due to a previous infection with remaining residual titer, with subsequent and recent invasion of the body by the virus producing complement-fixing antibodies; or (b) this animal obtained a recent infection, resulting in appearance of neutralizing antibodies, prior to those of complement-fixing ones.

Hodes *et al.*[3] found complement-fixing antibodies against Japanese B virus in significant titers in 6 of 9 normal horses bled during the Okinawa human encephalitis outbreak of 1945. According to our previous deduction mentioned above, these data indicate that there were extensive inapparent infections among horses during the epidemic season of 1945.

Sabin, Ginder, and Matumoto[2] tested 20 normal equine serums in Okayama and Tokyo Prefectures for complement-fixing antibodies during the summer of 1946, with

two specific positives for Japanese B virus. Nineteen of these 20 horses were also tested for neutralizing antibodies against Japanese B virus with 11 positive results, including the 2 horses with complement-fixing antibodies.

From our results, it is evident that this discrepancy of the incidence of complement-fixing and neutralizing antibodies might be explained by differences of duration of these antibodies in animals.

With regard to the low incidence of complement-fixing antibodies in this group of animals,[2] it is of interest to note that their observations and laboratory studies[4] in the Okayama area during the summer of 1946 indicated that there were no human cases of Japanese B encephalitis.

SUMMARY

In order to note the extent of dissemination of Japanese B virus among domestic animals in Japan, horses, cattle, sheep, dogs, cats, and rabbits were tested for neutralizing and complement-fixing antibodies periodically during the summer months of 1947.

1) Based on results of neutralization tests, inapparent infections of Japanese B virus during the summer of 1947 were confirmed in 14 of 15 horses in Okayama Prefecture, 5 of 6 cattle, and 1 of 8 sheep in Okayama Prefecture. Such infections were not demonstrated in 8 dogs, 10 cats, and 10 rabbits in Saitama Prefecture, nor in 9 rabbits in Tokyo. Although the results are not clear cut, there were probably no inapparent infections demonstrated in horses in Yokohama.

2) It was demonstrated that complement-fixing antibodies for Japanese B virus could be produced in horses and probably in cattle, as a result of inapparent infection by the virus.

3) Complement-fixing antibodies for Japanese B virus in horses appear to be of a transitory nature with a maximum duration of twelve to sixteen weeks. It is probable that a positive complement fixation titer means recent infection.

4) A rise in complement-fixing antibodies can be expected when an immune animal (based on preseason neutralization titers) is again exposed to the virus.

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Residual Arsenic and Strychnine in the Tissues of Drug-Treated Cattle

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THE OBSERVATIONS reported in this paper were made as one part of a comprehensive investigation, which was conducted during two consecutive years, into the prolonged administration of the so-called stimulant drugs to heifers. Inasmuch as the active ingredients of the two drugs studied were arsenic trioxide and strychnine, both of which are highly toxic, the possibility of accumulated residues of these substances in the tissues and organs of treated animals was considered a vital aspect of the study. The maximum allowable content of arsenic (reported as arsenic trioxide, As_2O_3) in food, as designated by the Pure Food and Drug Laws,[1] is 3.57 parts per million (p.p.m.), and it is obvious that the detectable presence of strychnine in food, even in minute quantities, should be considered dangerous.

The foremost purpose of this study, then, was to determine if residual amounts of arsenic in the tissues of animals administered arsenic trioxide over a prolonged period exceeded the maximum allowable tolerance, and if any trace of strychnine could be determined in the tissues of animals given nux vomica.

In addition, during the first year's trials, a rather complete blood study of the experimental animals was carried out simultaneously. This latter phase of the experiment included, in addition to the lots already mentioned, one group of animals which were receiving thiouracil to determine its effect upon growth rate and feed utilization. Assays for this drug, however, were not conducted because no satisfactory method had, at that time, been developed for its microdetermination in biological materials.

Groves, McCulloch, and St. John,[2] in experiments upon the effect of continued ingestion of spray residues by swine, reported an instance in which 1 animal was fed a total of 29.4 Gm. of pure arsenic trioxide during a period of 283 days, an average daily dose if about 0.1 Gm. per day. When autopsied at the end of this time, no significant pathologic changes were found. Increases, however, were noted in the arsenic content of the liver, heart, and spleen of the experimental animal when compared with levels found in the controls. A corresponding increase in the blood-arsenic of the treated animal was also reported. Small but measurable amounts of arsenic, an average of 0.43 p.p.m. as arsenic trioxide, were detected in the corresponding organs of the control animal in this experiment. According to Goodman and Gilman,[3] arsenic is only very slowly excreted in the urine and feces and may continue for as many as seventy days after repeated administration. According to Grollman and Slaughter,[4] arsenic stored in the tissues may be found there for a long time, even after it has disappeared in the feces and urine. These authors also state that no marked tolerance for strychnine is developed, and that the drug is readily destroyed in the body, mainly by oxidation in the liver. Urinary excretion of strychnine is generally complete within ten hours.

EXPERIMENTAL

The drugs under study were administered at the following levels: arsenic trioxide, 18 Gm., and nux vomica, 300 Gm. per 1,000 lb. of grain ration. Based upon an average grain consumption for both years, these levels corresponded to average daily dosages of 0.357 and 5.69 Gm., respectively, of the two drugs. The drug trials, during 1946 and 1947, were of 120 and 201 days duration, respectively.

Tissues were obtained for assay both by biopsy from the biceps femoris or semitendinosus muscles and from the carcasses of slaughtered animals. During 1946, biopsies were taken from the biceps femoris immediately after the end of the trial and again from the semitendinosus muscle of the same animal after a twenty-day depletion period. In 1947, biopsies from the biceps femoris muscle were analyzed at the end of the trial only.

Test samples were removed from the following organs and muscular parts of the carcasses after slaughter: liver, heart, kidney, eye, muscle over the twelfth rib, sirloin tip, and foreshank. In 1946, all animals were slaughtered following a 41-day drug depletion period. In 1947, samples were secured from the carcasses of 2 animals in each lot slaughtered immediately at the end of the drug trial period. All samples were frozen immediately after being received at the laboratory.

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Blood samples for chemical analyses and differential cell counts were taken during the 1946 trials only. They were obtained thirty days after the initiation of the drug feeding, at the end of the trial, and at the end of a thirty-day drug depletion period. The animals were bled from the jugular vein. Tubes of oxalated and unoxalated blood were drawn at each bleeding, for the determination of carotene, vitamin A, ascorbic acid, serum calcium and phosphorus, and blood glucose. Hemoglobin, red cell counts, and differential white cell counts were also carried out on specimens and films taken at the same time.

Preparation of Tissue Samples for Assay.—All visible fat was removed and the lean tissue was finely chopped in preparation for either the extraction process used in the detection of strychnine or for digestion prior to the determination of arsenic. The combination nitric acid-sulfuric acid method was employed for digestion of the arsenic test samples. Twenty-five to 30 Gm. of the undried tissue were used in both the arsenic and strychnine assays.

Analytical Methods.—Arsenic.—Because of the small amount of tissue obtained, especially in the case of the biopsies, the method of Gutzheit lacked sensitivity in the range of the total arsenic contained in the samples. For this reason, the microcolorimetric procedure developed by Sandell[5] was used. This procedure employs the same basic principle for separation and isolation of the arsenic as arsine as other common methods. The arsine is, in turn, absorbed, oxidized, and determined colorimetrically by use of a molybdate reagent. During the 1946 trials, part of the samples were assayed by both the Sandell and the Gutzheit[6] methods. These determinations were made in two independent laboratories by different persons and were intended as checks upon the analytical results. In most cases the results checked well, although those obtained by the latter method tended in some cases to be somewhat higher. As has been previously stated, however, the amount of arsenic present in the samples used was below the range for which accurate measurement could be made by the Gutzheit procedure.

Strychnine.—The determination was qualitative only. No effort was made to separate impurities of brucine, probably present in the crude drug, inasmuch as both principles were considered to be highly toxic. The extraction procedure was the same as that given by the Tentative and Official Methods of the A.O.A.C.[6] Aliquots of the final extracts from this procedure were evaporated to dryness and tested for the presence of strychnine by means of a drop of concentrated sulfuric acid and a small crystal of potassium dichromate. Although the procedure was qualitative only, it was shown to be sensitive to very small amounts of the alkaloid, which fully served the purpose of the assay. The distinctive coloration produced in the positive test was easily obtained for comparison by evaporating a minute amount of pure strychnine sulfate in chloroform to dryness and conducting the final test upon the residue.

Blood Assays.—Standard clinical methods were used in all cases. The titration method of Shafer and Somogyi,[7] employing Somogyi's sugar reagent No. 50, was employed in measurements of blood glucose. Ascorbic acid, carotene,[8] and vitamin A[9] were measured in the plasma by means of the standard colorimetric methods. An Evelyn photoelectric colorimeter was used for these determinations. Serum calcium analyses were made by titration with 0.01 normal potassium permanganate after acidification of precipitated (centrifuged) calcium oxalate. The colorimetric reaction of Fiske and Subbarow[10, 11] was used for the measurement of serum phosphate.

Hemoglobin.—The oxyhematin method was employed using a blood sample of 0.02 cc. Di-

TABLE I—Arsenic Analyses of Biopsies, Internal Organs, and Muscle Tissues

Year of trial	Treatment	Time of sampling	No. animals included in average	p.p.m. As_2O_3 Biopsy	p.p.m. As_2O_3 Liver		p.p.m. As_2O_3 Heart		p.p.m. As_2O_3 Kidney	p.p.m. As_2O_3 Sirloin tip		p.p.m. As_2O_3 Over 12th rib	p.p.m. As_2O_3 Foreshank
				S.	S.	G.	S.	G.	S.	S.	G.	S.	S.
1946	Fowler's	End of trial	2	4.21									
1947	Fowler's	End of trial	2	12.67									
1946	Fowler's	20-day depl.	2	0.71									
1947	Control	End of trial	3	0.20									
1947	Fowler's	End of trial	2		2.22		2.09		2.29	2.81		1.64	2.43
1947	Control	End of trial	2		0.53		0.21		0.46	0.44		0.55	0.66
1946	Fowler's	41-day depl.	5		0.42		0.40	1.60	0.38	0.59	1.60	0.12	0.49
1946	Control	41-day depl.	6		0.15		0.34	0.95	0.14	0.59	1.60	0.12	0.69

S. = Sandell. G. = Gutzheit.

lute ammonium hydroxide was used for its formation.

Blood Counts.—Red blood cell counts were run by the standard method, using Heayem's solution as diluent. Wright's stain was used for the differential white cell counts.*

RESULTS

No traces of strychnine could be detected in any of the samples analyzed. This is in accordance with the generally accepted view that the alkaloid is very rapidly eliminated from tissues and is rarely, if ever, found to be present outside of the digestive tract, even following the ingestion of fatal doses.

Results of the arsenic determinations are shown in table 1. The data from both years' trials have been combined and are given as the average values for the group. With the exception of the biopsy samples taken immediately at the end of the drug trials, the arsenic content (calculated as arsenic trioxide) of the tissues and organs analyzed, fell below the 3.57 p.p.m. maximum allowed by the Pure Food and Drug regulations. The biopsy samples, however, taken in both years immediately after the end of the drug trials, contained arsenic at levels which were in excess of this specified amount. It was speculated that high levels of arsenic found in the biopsies were possibly due to an excessively high blood level which was eliminated by bleeding during slaughter. Unfortunately, arsenic analyses could not be run upon the blood of these animals. This aspect warrants further study.

From the combined data of both trials, it is also seen that there was a decrease in the arsenic content with increasing length of the period between cessation of drug administration and slaughter. The highest results were obtained from animals slaughtered immediately after termination of the trial periods, while those of animals slaughtered after a 41-day depletion period, and even biopsies from animals after a twenty-day depletion period, did not differ significantly from those of the control animals. It is interesting to note that, as was previously stated in connection with the work of Groves *et al.*,[2] small but measurable amounts of arsenic were found in the tissues of the control animals. It is not known whether this small content is inherent or if it was the result of the constant ingestion and accumulation of small amounts of the element present in the normal rations of the animals.

Blood data from the 1946 trials showed some variation between groups in the levels of carotene and blood sugar, but the differences were considered to be within the normal range of fluctuation expected in the blood concentration of these substances. Likewise, all cell counts from both treated and control animals were within the limits of expected normal values and did not differ significantly between groups.

SUMMARY AND CONCLUSIONS

The results obtained under the conditions of these trials indicate that strychnine is very rapidly eliminated from the tissues and organs of animals fed continuous sub-lethal doses of nux vomica. No trace of the alkaloid could be detected by the method used, even in biopsy samples removed from animals immediately after cessation of drug treatment. Arsenic was also depleted from the tissues of animals after withdrawal of the drug. However, the rate of depletion was slower inasmuch as residues of the element were found in samples from animals slaughtered immediately after the trials in amounts which were above normal and which, in some cases, at least approached the maximum permitted by law. After a depletion period of forty-one days, however, arsenic residues did not differ significantly from levels found in the corresponding samples taken from control animals. The arsenic content of biopsies taken from animals during or immediately after the actual drug period were in excess of the limit specified as dangerous, but corresponding samples from live animals after a twenty-day depletion period did not differ in their arsenic content from those taken from control animals. Biopsies taken from animals immediately after cessation of drug treatment, in all cases, contained more arsenic than tissues from animals slaughtered at the same time. The explanation for this difference was not determined.

None of the drugs tested had any apparent effect upon the blood picture of the treated animals, under the conditions of the trials, beyond that expected within the range of normal variation.

From the data obtained, it is concluded that, while the presence of dangerous residues of the toxic ingredients in the meat of animals previously administered solutions containing arsenic (such as Fowler's solution) as a stimulant is not great, a depletion period between cessation of treatment and slaughter should be advocated and recommended. This precautionary practice is indicated especially in the case of arsenic, inasmuch as meat from animals treated

*The blood counts were conducted by Dr. D. R. Cordy, associate professor of veterinary pathology, College of Veterinary Medicine, State College of Washington, with the technical assistance of Miss Irene Woods.

with this drug and slaughtered immediately after the end of the drug trials contained levels of residual arsenic which approached the maximum amount allowed by the Pure Food and Drug laws.

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Dosage of Caronamide Required for Inhibition of Renal Tubular Excretion of Penicillin in Horses

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WHEN ADMINISTERED parenterally in aqueous solution, penicillin is eliminated rapidly from the body, primarily by renal excretion, with the antibiotic falling to undetectable levels in the plasma in two or three hours.[1, 2] The rapidity of excretion has been explained by showing that penicillin is excreted by both glomerular filtration and tubular excretion, with approximately 20 per cent being filtered at the glomeruli and 80 per cent eliminated by the renal tubules.[3] For reducing the excretion of penicillin by the renal tubules and thereby increasing plasma levels, diodrast[4] and para-aminohippuric acid[5] have been used. The limitation of these two compounds for clinical use has been described.[3, 6]

In introducing a new compound, caronamide,* for suppressing renal elimination of penicillin,† Beyer[3] described the properties desired in such an agent and suggested a "new concept of competitive inhibition of the renal tubular excretion of penicillin." The action was thought to be one of substrate competition between caronamide and penicillin, resulting in inhibition of excretion of penicillin by the tubules.

In another investigation,[6] it was found that various doses of caronamide would partially, or completely, suppress the tubular excretion of penicillin administered by various routes. Its action on the kidney tubules was completely reversible, with normal elimination of penicillin occurring several hours after termination of caronamide administration.

In the dog, it has been determined that caronamide is excreted by glomerular filtration and is refractory to excretion by the renal tubules.[7] Its competitive action is effective against penicillin, phenolsulfonphthalein, and para-aminohippuric acid. With sufficient dosage of caronamide, penicillin excretion was limited to glomerular filtration. The Tm** of glucose or arginine, and the clearances of urea, sulfonamide, and creatinine were not affected. Doses that completely suppressed tubular excretion of penicillin did not significantly affect the heart rate, respiratory rate, systemic blood pressure, or kidney volume.

TABLE I—Average Serum Levels of Penicillin Obtained with Various Doses of Caronamide

No. of determinations	Caronamide Gm./100 lb. intrav. at zero hr.	Caronamide Gm./100 lb. orally at zero hr.	Caronamide Gm./100 lb. orally at 3rd, 6th hr.	Average levels of penicillin per cc. of serum at specified hours following administration of penicillin and caronamide							
				1	2	3	4	5	6	7	8
6	2	–	2	1.17	0.42	0.07	0.016	0.002	0.00	0.00	0.00
6	4	–	4	2.13	0.96	0.20	0.03	0.007	0.00	0.00	0.00
6	4	2	2	2.34	1.60	0.53	0.21	0.08	0.04	0.013	.....
6	4	4	4	4.26	2.13	1.01	0.49	0.24	0.12	0.07	0.036
6	3	3	3	1.60	0.48	0.12	0.019	0.007	0.00	0.00	0.00
6	4	3	3	2.56	2.13	0.96	0.32	0.10	0.04	0.002	0.00
4	6	6	6	2.56	2.24	1.04	0.34	0.07	0.018	0.003	0.00
4	4	4	4	1.28	0.48	0.12	0.01	0.00	0.00	0.00	0.00
4	4	4	4	2.24	1.12	0.36	0.08	0.025	0.005	0.00	0.00
24	Aqueous penicillin controls			0.66	0.36	0.06	0.019	0.005	.....	.....	.....

METHODS

Under practical conditions, it would be desirable to have plasma concentration of caronamide sufficient to inhibit renal excretion of penicillin at the beginning of treatment. For this purpose, caronamide was administered intravenously, or both intravenously and orally, immediately before injection of penicillin, with subsequent oral administrations at three-hour intervals.

Solutions for intravenous injection, contain-

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*Caronamide, 4' carboxyphenylmethanesulfonanilide, sold under the trade name "staticin", was supplied for experimental investigation by courtesy of Sharp and Dohme, Inc., Glenolden, Pa.

†The penicillin used in this investigation was supplied by courtesy of Schenley Laboratories, Inc., New York, N. Y.

**The maximal rate of reabsorption of glucose, or glucose Tm, amounts to about 320 mg. per minute in man. It is determined by raising the plasma-glucose concentration and measuring simultaneously the rate of filteration and the excretion of sugar in the urine.

ing 33.33 per cent caronamide, were prepared according to a method recommended by Wilcox.[8] Oral doses were administered by stomach tube. Suspensions for oral administration containing 20 per cent caronamide were made by mixing the powdered compound with 0.25 per cent solution of agar. To determine the dosage of caronamide required for maintenance of the inhibitory effect, the compound was administered at three-hour intervals, with the penicillin injection being given immediately following the third dose of caronamide. The means of establishing caronamide dosage has been indirect, through determining the optimum blood level response of fixed doses of penicillin with various doses of caronamide.

Penicillin was administered by intramuscular injection, using doses of 500 units per pound of body weight in all dosage trials. The penicillin was the potassium salt of crystalline penicillin G. Blood samples for assay were obtained from the jugular vein at the first hour following injection, and at hourly intervals thereafter. The method of assay was the serial dilution method reported previously.[9] Caronamide doses for both oral and intravenous administration were measured in grams per 100 pounds of body weight. All horses used in these experiments were apparently normal and had free access to feed and water during the test periods. Except for one determination, the same horses were used for all dosage trials.

RESULTS

Aqueous Controls.—For comparison with results obtained through simultaneous use of penicillin and caronamide, the average serum level response obtained in 24 dosage trials using potassium crystalline penicillin G was used.[10] In this series of trials, all doses were administered by deep injection into the musculature of the neck. The solutions contained 10,000 units per cubic centimeter and the dosage was 500 units per pound of body weight in all trials. The duration of the serum level response varied from two hours in young foals to five hours in yearlings and adults. An average of the serum level responses obtained in this study is included in table 1.

Throughout the course of this study, the dosage trials were performed in the sequence of the order of listing for the serum level response averages and enhancement values given in tables 1 and 2. As the dosage of caronamide was increased, there was a general trend toward an increase in serum level values and in duration of the serum level response for the first four dosage trials. The advantage obtained with coadministration of penicillin and carona-

TABLE 2—Enhancement Effect Obtained with Coadministration of Penicillin and Caronamide

No. of determinations	Caronamide Gm./100 lb. intrav. at zero hr.	Caronamide Gm./100 lb. orally at zero hr.	Caronamide Gm./100 lb. orally at 3rd, 6th hr.	Enhancement effect in multiples of average serum levels of aqueous controls at hourly periods following injection of penicillin 1	2	3	4
6	2	–	2	1.7	1.1	0.0	0.0
6	4	–	4	3.2	2.6	3.3	1.5
6	4	2	2	3.7	4.6	8.9	11.0
6	4	4	4	6.4	5.9	16.8	25.2
6	3	3	3	2.4	1.3	2.0	0.0
6	4	3	3	3.8	5.9	16.0	16.7
4	6	6	6	3.8	6.2	17.3	17.8
4	4	4	4	1.9	1.3	2.0	0.0
4	4	4	4	3.4	3.1	6.0	4.1

TABLE 3—Caronamide 4.0 Gm./100 lb. Intravenously and 4.0 Gm./100 lb. Orally at Zero Hour Followed by Four Oral Doses of 4 Gm./100 lb. at Three-Hour Intervals. Penicillin 500 Units/lb. Intramuscularly, Aqueous Solution, Administered Six Hours Following First Dose of Caronamide

Weight (in lb.)	Penicillin dose (units)	Serum levels at specified hours following injection of penicillin 0	1	2	3	4	5	6	7	8
500	250,000	–	0.64	0.16	0.02	0.015	0.015	0.00	0.00	0.00
480	240,000	–	0.64	0.32	0.08	0.02	0.015	0.00	0.00	0.00
500	250,000	–	0.64	0.32	0.16	0.04	0.02	0.02	0.00	0.00
380	190,000	–	1.28	0.32	0.08	0.02	0.015	0.00	0.00	0.00
450	225,000	–	0.64	0.16	0.04	0.02	0.02	0.015	0.00	0.00
500	250,000	–	0.64	0.32	0.08	0.01	0.007	0.00	0.00	0.00
480	240,000	–	0.64	0.32	0.16	0.02	0.010	0.01	0.007	0.00
500	250,000	–	0.64	0.32	0.16	0.02	0.010	0.01	0.01	0.00
380	190,000	–	0.64	0.16	0.01	0.007	0.007	0.00	0.00	0.00
450	225,000	–	0.64	0.16	0.16	0.02	0.007	0.007	0.00	0.00
Average serum level		–	0.70	0.25	0.095	0.019	0.013	0.006	0.002	0.00
Caronamide administration		3rd			4th			5th		
Hours of caronamide administration		6	7	8	9	10	11	12	13	14

mide is also evident in the enhancement multiples for the serum level averages given in table 2. In the fourth dosage trial, caronamide doses of 4 Gm. 100 lb. orally and intravenously, immediately preceding intramuscular injection of penicillin and two subsequent doses of 4 Gm. 100 lb. at three-hour intervals, resulted in enhancement multiples of 6.4, 5.9, 16.8, and 25.2 for the first four hours of the test period. The duration of the serum level response was from one to three or four hours longer than obtained with the aqueous penicillin controls. In two subsequent trials, the dosage of caronamide was reduced 25 per cent and the enhancement effect was lowered approximately 50 per cent, or more, for respective assay periods. There was also reduction of the duration of the measurable serum level period.

Following completion of the first six dosage trials, there was an interim of several months during which the group of horses received no caronamide. Upon resumption of the work, two trials for determining the maintenance dosage requirement were made. In one of these (table 3), initial oral and intravenous caronamide doses of 4.0 Gm./100 lb., followed by two oral doses of 4 Gm./100 lb. at three-hour intervals, were given with the penicillin injection following the third oral dose. Two oral caronamide doses of 4.0 Gm./100 lb. were given subsequent to injection of the penicillin. This dosage schedule produced no significant effect on the serum level response.

Increasing the oral dose of caronamide to 8 Gm./100 lb. at three-hour intervals, with three doses preceding and two doses following injection of penicillin, produced no significant improvement in the serum level response (table 4). The failure to obtain satisfactory serum level responses from the maintenance dosage trials suggested that this group of horses may have acquired an increased facility for elimination of caronamide. In investigating this possibility, a caronamide dosage schedule of 6 Gm. 100 lb. orally and intravenously, immediately preceding the penicillin injection with two subsequent oral doses of 6 Gm. 100 lb. at three-hour intervals, was used. Results obtained with this dosage schedule were inferior, in both enchancement multiple and duration of the serum level responses, to those obtained earlier with 4 Gm. 100 lb. in the same schedule (tables 1 and 2). The experiment was repeated, using caronamide doses of 4 Gm. 100 lb. with the same schedule. In these trials, the enhancement values were one third or less than those obtained earlier with the same dosage schedule, and the duration of the serum level response was approximately 50 per cent shorter (tables 1 and 2). The same dosage schedule with 4 Gm. of caronamide 100 lb. was then given to horses which had not previously received carona-

TABLE 4—Caronamide 8 Gm./100 lb. Orally at Zero Hour Followed by Four Oral Doses of 8 Gm./100 lb. at Three-Hour Intervals. Penicillin 500 Units/lb. Intramuscularly, Aqueous Solution, Administered Six Hours Following First Dose of Caronamide

Weight (in lb.)	Penicillin dose (units)	Serum levels at specified hours following injection of penicillin								
		0	1	2	3	4	5	6	7	8
505	252,500	–	0.64	0.32	0.08	0.015	0.00	0.00	0.00	0.00
507	285,000	–	0.64	0.32	0.04	0.015	0.00	0.00	0.00	0.00
420	210,000	–	1.28	0.64	0.16	0.02	0.00	0.00	0.00	0.00
530	265,000	–	1.28	0.64	0.16	0.04	0.015	0.00	0.00	0.00
Average serum level		–	0.96	0.48	0.11	0.02	0.003	0.00	0.00	0.00
Caronamide administration		3rd			4th			5th		
Hours of caronamide administration		6	7	8	9	10	11	12	13	14

DISCUSSION

The enhancement of the serum concentration of penicillin has been of sufficient degree to indicate that caronamide may have a useful place in veterinary therapeutics for establishing high serum concentrations at the initiation of treatment. For this purpose, it appears that the dosage of caronamide for horses should be 4 to 6 Gm./100 lb. of body weight both intravenously and orally, immediately preceding

the injection of penicillin. By coadministration of caronamide and penicillin, it appears possible to expand the usefulness of the antibiotic by obtaining blood levels effective against organisms with penicillin resistance sufficiently high to be unaffected by ordinary means of administration. Successful clinical use of large doses of penicillin at infrequent intervals for a number of conditions has been reported.[11] In view of these findings, it is possible that large doses of penicillin and caronamide given simultaneously two or three times daily may prove of value in veterinary practice.

In horses having their first experiences with the compound, enhancement of the serum levels of penicillin with caronamide doses in the optimum range of 4 to 6 Gm./100 lb. was approximately threefold to sixfold for the first and second hours, sixfold to sixteenfold for the third hour, and fourfold to twenty-five fold for the fourth hour following the injection of penicillin. Penicillin serum levels of 1.28 to 5.12 units per cubic centimeter were obtained for the first hour following injection, with a gradual falling throughout the measurable serum level period.

SUMMARY

Data on the influence of various doses of caronamide upon serum levels of penicillin have been presented.

The initial dosage of caronamide required for optimum effect on the serum level response appears to be 4 to 6 Gm./100 lb. of body weight intravenously and orally, immediately preceding injection of penicillin.

The maintenance dosage requirement has not been established.

The value of caronamide as an adjunct to penicillin therapy has been discussed.

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The Virus of Equine Encephalomyelitis in the Peripheral Circulation of Guinea Pigs and Rabbits Following Artificial Inoculation

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IN THIS LABORATORY, early attempts to transmit the virus of equine encephalomyelitis from animal to animal through the agency of arthropod vectors yielded inconclusive results. The explanation seemed to lie in the fact that the amount of virus varied from time to time in the blood stream of the host animal. Therefore, an endeavor was made to determine the period of time when the virus concentration was greatest in the peripheral circulation.

Shahan and Giltner[1] successfully recovered the virus from the blood of experimentally inoculated horses, through the medium of guinea-pig inoculation, twenty-four, thirty-six, and forty-eight hours after the horse had been subcutaneously inoculated. This interval corresponds, in a measure, with the febrile period in this animal. It seemed desirable to determine whether a similar condition existed in the laboratory experimental animals. This was particularly important in the case of such intermittent feeders as flies and mosquitoes which engorge in a few minutes and must be fed when the virus is readily available in the peripheral circulation.

The periodic concentration of virus is of lesser importance in the case of ticks, which remain attached to the animal for much longer periods of time. Blood is sucked at intervals of a few hours throughout this time, so that the virus, if present, will be engorged by the tick. The only conceivable complication to tick infection by this method would lie in the effect of any immunity products which might be ingested with the blood and cause a preclusion of the virus from the tissues in the body of the tick. Thus, the knowledge of the time of virus concentration would be advantageous in placing vectors upon host animals, so that the virus content would be at the maximum and immunity products at a minimum.

The course of the disease following various routes of inoculation is important because the length of the period between inoculation and death may in 1 case allow ample time for mosquitoes or flies to engorge virus, but be insufficient in the case of ticks. Death usually follows intracranial inoculation of guinea pigs in from four to five days. This period is too short to allow the adults of *Dermacentor variabilis*, *Dermacentor andersoni*, and *Rhipicephalis sanguineus* to properly complete their engorgement. It was necessary to adopt a route of inoculation which would prolong the course of the disease and still retain a sufficient concentration of virus in the blood to result in a high mortality. The intraperitoneal route satisfied these requirements better than the subcutaneous route, although the two follow parallel courses. A strain of Western equine encephalomyelitis virus was fixed, by repeated passage, to kill

TABLE I—Postinoculation

Master pig	No.		Hour blood drawn and injected			
			8	24	48	72
Subcutaneous inoculated	32	Guinea pig	R-16 days	R-20 days	D-11 days	R-20 days
		Egg	D-18 hr.	Hatched	D-18 hr.	D-24 hr.
	37	Guinea pig	R-16 days	D-20 days	D- 6 days	R-20 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	Hatched
	42	Guinea pig	R-16 days	R-20 days	R-20 days	R-20 days
		Egg	D-18 hr.	D-18 hr.	D-24 hr.	D-24 hr.
Intraperitoneal inoculated	47	Guinea pig	R-20 days	D- 5 days	D- 6 days	R-20 days
		Egg	D-18 hr.	D-18 hr.	D-36 hr.	D-48 hr.
	52	Guinea pig	R-20 days	R-20 days	D-20 days	R-20 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	Hatched
	57	Guinea pig	R-20 days	R-20 days	R-20 days	R-20 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	Hatched

R = Released. D = Died. All guinea pigs were inoculated intraperitoneally except 32, 37, and 42. All master pigs died, following typical symptoms, in from five to eight days.

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with considerable regularity following intraperitoneal inoculation. This virus was used in the present work.

LABORATORY PROCEDURE

Healthy guinea pigs, weighing approximately 600 Gm. and possessing unscarred ears, were selected for the host animals. Samples of blood were taken from the ear veins. There were several reasons for selecting ear veins as a source of blood supply: easy accessibility to the peripheral circulation and the belief that vectors choose the ear because of the sparse hair growth and vascularity of this appendage. Three guinea pigs were inoculated intraperitoneally and 3 others subcutaneously with a 0.5-cc. dose of 1 per cent guinea-pig, virus-infected brain suspended in physiologic saline. Samples of blood were collected in a tube containing sodium citrate from each of the above animals at eight, twenty-four, forty-eight, seventy-two, ninety-six, and one hundred and twenty hours following inoculation. Approximately 0.2 cc. of the blood thus collected was injected intraperitoneally into guinea pigs, weighing 300 Gm., immediately after it was obtained. The intraperitoneal route was adopted here to conform to the method used to infect the primary animals and because the intracranial route was attended with considerable shock, which in several instances was sufficient to kill the animal. Each guinea-pig injection was paralleled by inoculating 0.1 cc. of the citrated blood into a 9-day incubated chicken embryo using the Burnet[2] modification of the Woodruff-Goodpasture[3] technique. The guinea pigs were observed and temperatures taken morning and evening for sixteen to twenty days, at which time the surviving animals were released. The inoculated eggs were candled twice daily until they hatched or the embryo died.

In order to confirm the results, the experiment was repeated. Two rabbits were added to the group of primary animals; one of these received an intraperitoneal inoculation and the other a subcutaneous inoculation.

Table 1 shows the data on two groups of 3 guinea pigs, each inoculated either intraperitoneally or subcutaneously, and the effect of blood drawn eight, twenty-four, forty-eight, and, seventy-two hours later when injected into young guinea pigs and chicken embryos.

Table 2 shows the data on 2 additional guinea pigs, the first receiving an inoculation by the subcutaneous route and the second by the intraperitoneal method. In addition, 1 rabbit was added to each of the guinea-pig groups.

TABLE 2—Postinoculation

Master pig	No.		Hour blood drawn and injected					
			8	24	48	72	96	120
Subcut.	73	Guinea pig	R- 8 days	D-13 days	D- 7 days	R-17 days	R-16 days	R-16 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	D-18 hr.	Hatched	Hatched
Intraper.	74	Guinea pig	R-20 days	D-12 days	R-18 days	D- 8 days	R-16 days	R-16 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	D-18 hr.	D-18 hr.	Hatched
Rabbit								
Intraper.	351	Guinea pig	D- 9 days	D-10 days	D- 5 days	R-17 days	R-16 days	R-16 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	Hatched	Hatched	Hatched
Subcut.	352	Guinea pig	R-20 days	D-12 days	D- 6 days	R-17 days	R-16 days	R-16 days
		Egg	D-18 hr.	D-18 hr.	D-18 hr.	Hatched	Hatched	Hatched

Both master guinea pigs and both rabbits died, following typical symptoms, in from five to eight days.

SUMMARY

1) Beginning at the eighth hour and extending beyond the seventy-second hour but disappearing by the 120th hour, following subcutaneous and intraperitoneal inoculation of brain suspensions containing the virus of equine encephalomyelitis, virus was found in the ear blood of guinea pigs and rabbits in sufficient quantities to kill 9-day-old chicken embryos when 0.1 cc. of citrated blood was inoculated into the chorioallantoic sac.

2) In the above inoculated guinea pigs and rabbits, sufficient virus to kill guinea pigs upon intraperitoneal inoculation did not appear in the ear blood until the twenty-fourth hour and then persisted beyond the forty-eighth hour but, with one exception, disappeared by the seventy-second hour.

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Alpha-Naphthyl Thiourea (ANTU) Poisoning in Dogs

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ALPHA-NAPHTHYL THIOUREA (ANTU) has been recommended as an effective and specific poison for the common (Norway) rat.[1] ANTU has been used in several localities in the United States in individual or community rat-exterminating campaigns. In several such campaigns, not only rats but also dogs were killed by the effects of the poison. The deaths of dogs arose from owners' carelessness in releasing them to run while poisoned bait or poisoned rats were available and from too little emphasis upon the toxicity of ANTU for dogs.

In Ames, Iowa, a rat-extermination program was launched on a community-wide basis late in 1946. Three per cent ANTU was mixed with ground horsemeat for bait. A tablespoonful, or about 100 Gm., of bait was dispensed early in the evening and supposed to have been collected early in the morning. This was repeated for three nights. People were warned not to allow their pets to roam. However, the general impression was conveyed that ANTU was relatively harmless to dogs because it stimulated vomition when ingested. It was assumed that by vomiting, the dog would rid itself of any poisoned bait that it might have eaten. Since rats cannot vomit, ANTU would be fatal to rats.

The day after the poisoned bait was set out, the first of a series of sick dogs was presented to the Stange Memorial Clinic of Iowa State College. During the next two weeks, 23 dogs were received showing symptoms of ANTU poisoning. A total of 34 dogs poisoned by ANTU were brought to the Clinic by their owners during the two months following the campaign. All but 3 of the patients died. Many unclaimed dogs were found dead or moribund by the city employees.

Part I*

CLINICAL ASPECTS

The clinical aspects of poisoning by alpha-naphthyl thiourea (ANTU) are manifested characteristically and differently from those of any other chemical poison commonly encountered.

The symptoms are peracute, acute, or subacute; no cases yet observed have become chronic. The poisoned animal stands or sits on its haunches as long as it can and when weakness makes this impossible, it assumes a sternal recumbent position, many times dying in this position.

The onset of symptoms is characterized by vomition, the vomitus including the gastric ingesta, frothy mucus, perhaps streaked with blood, and at times small, castlike shreds of mucous membrane. The breathing becomes rapid with short, jerky inspirations that do not fill the lungs. Expiration is accompanied by a shallow, hacking cough. The legs, ears, and nose are cold to the touch, as are the lips and gums. The mucous membranes of the mouth, tongue, and conjunctivae are cyanotic and anemic. As the condition progresses, the peripheral pulse becomes imperceptible, while the cardiac impulse, as felt on the chestwall, is accelerated, weak, and floundering. With stethoscope or phonendoscope, the heart sounds cannot be distinguished but the listener is impressed by the extreme exertion of the heart. Severe pain is demonstrated by grunts and whines when the animal is palpated over the gastric area.

When presented for treatment, about one-half of our patients had normal temperatures and the remainder had subnormal readings, some being as low as 97 F. The subnormal temperature is a fairly good index that the patient will die. It is significant that in those few cases that recovered, the temperature was never below 100 F. As death approaches, the temperature falls still lower and has been observed to reach 94 F., or below the graduations on the ordinary thermometer.

The gastrointestinal symptoms become exaggerated. Patients continue to vomit throughout the course of the disease. The vomitus becomes bloody, perhaps stained with bile, and may be practically whole blood, resembling the hemorrhagic vomitus of acute arsenic poisoning. A diarrhea develops; the first feces passed are normal, then change to a liquid consistency, sometimes with streaks of blood. If the patient lives long enough, the feces become hemorrhagic and very watery.

As the terminal stage approaches, weakness is more manifest and the dyspnea more extreme; attempts to cough are more and more feeble. Moist râles, which could be heard earlier, now disappear. Percussion, if performed early, will show areas of dullness, but later the areas coalesce, indicating

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*Part 1 was prepared by Dr. Dwight A. Smith.

that the lungs and thoracic cavity are congested, edematous, and filled with fluid.

When the illness has progressed to this stage, the animal is almost or completely comatose and does not respond to external stimuli. Even during the stages preceding coma, the animal allows the tongue to fall from the mouth. Fluid from the lungs escapes from the mouth, some of which the animal may attempt to swallow. If this is accomplished, it is regurgitated, stained with blood from the gastric wall. This fluid may continue to escape from the mouth for some time after death.

Most of the deaths occur in two to four hours after symptoms appear; many patients die within one hour. A few that have ingested small amounts of poison live eight to twelve hours, but seldom did one of our patients live longer than twelve hours.

The symptoms are usually sufficient to differentiate ANTU from other chemical poisonings, but the diagnosis is confirmed by a combination of history, symptoms, pathology, and chemical analysis.

The history of a rat-eradication campaign should cause the veterinarian to make an investigation as to what lethal agent was used to destroy the rats. Some agriculturalists have used ANTU without knowing how toxic the compound is to animals other than rats. There probably have been instances of malicious use of ANTU since the public has learned how lethal it may be to the neighbor's dog.

The prognosis should always be considered unfavorable. A few dogs known to have ingested ANTU were treated by immediate evacuation of the stomach with apomorphine sulfate (1/10 to 1/20 gr.), and no symptoms developed. Of 34 dogs showing symptoms of ANTU poisoning which were treated at the Stange Memorial Clinic of Iowa State College, 3 recovered, probably because the amount ingested was sublethal. It is probable that a few dogs ingest a small amount of the poison when the stomach is empty, regurgitate voluntarily, and recover.

Part 2**

POSTMORTEM PATHOLOGY

When poisoning occurs, the necropsy findings are sufficiently characteristic so that a diagnosis can be made on the basis of them. This is especially fortunate since body fluids suitable for chemical tests often are not obtainable; and at the time of most of our cases, no chemical test was known.

The first conspicuous change to be noticed at autopsy is hydrothorax. This condition was present in all the 46 cases here reported, although in 6 of the experimental dogs it was recorded as slight. Ordinarily, the change is extremely marked. As soon as the sternal plate is removed, the thoracic cavity is seen to be full or half full of watery fluid, which is clear and colorless until contaminated by escaping blood.

In addition to considerable hyperemia of the tracheal mucosa, extremely severe edema of the lungs was present. With regard to this lesion, there was one exception among the 46,—an experimental dog which received an intraperitoneal injection of the material (the only dog in which it was so administered). The edema was recorded as slight in 2 natural and 3 experimental cases. In the remaining cases, large amounts of watery or blood-tinged fluid could be pressed from the cut surface of the lungs. In about one-third of the animals, there was so much fluid in the lungs that it flowed spontaneously from the severed trachea and it was not unusual for the animal to be found lying dead with a small pool of the pulmonary fluid at its muzzle. With the edema, there was congestion in 41 cases (90%), resulting in an irregular, reddish mottling of the pulmonary surfaces. In one of the 41 cases, beginning pneumonia was also recorded.

Edema of the mediastinal tissues, as well as of the lungs, was a noticeable feature in approximately 40 per cent of the cases.

Microscopic examinations of the lungs were made in 4 cases. The findings were severe, acute passive congestion, differing from the active hyperemia encountered in connection with inflammatory disease by the large number of greatly congested veins; diapedesis of erythrocytes into the alveoli and bronchi in some individuals; edema, usually leaving only a small precipitable residue; alternating atelectasis and emphysema; and rarely a leucocyte or two in an alveolus.

The poison is moderately irritant to the mucosa of the alimentary tract, although the lesions here were rather variable, influenced probably by lavage and other treatments in certain individuals. The lesion in the stomach was a catarrhal gastritis in the fundic region, recognized grossly by a diffuse reddening of greater or lesser severity. It was classified as severe in 25 (54%), moderate in 15 (32%), slight in 4 (8%), absent in 2 (5%), and unrecorded in 1 case. With 2 exceptions, the stomachs were empty, doubtless as the result of vomition.

Microscopic examinations were made of four stomachs. In addition to hyperemia, they all showed considerable exudation of mucus. The epithelium on the surface was

**Part 2 was prepared by Dr. Hilton A. Smith.

intact. The parietal cells seemed unusually numerous and prominent in all cases, and the chief cells seemed to have disappeared from the distal third of the majority of the gastric glands. We have no explanation of this phenomenon, but careful comparison with other canine stomachs confirmed the observation.

The small intestine usually showed acute catarrhal enteritis recognized grossly by redness, edematous swelling, and a small amount of exudative material. Among the 46 animals, enteritis was recorded as severe in 26 (57%), moderate in 14 (30%), slight in 4 (8%), and absent in 2 (4%). Frequently, the duodenal end of the gut was more severely involved than the ileal. In the experimental dogs which received the ANTU intravenously or intraperitoneally, the enteritis was rated as of moderate degree.

Microscopically, as determined from 4 cases, the enteritis was characterized by hyperemia, edema of the villi and lacteals, necrosis and desquamation of the superficial tips of the villi, and a heavy infiltration of white cells, mostly lymphocytes.

A feature believed of considerable diagnostic importance was the presence of large amounts of fresh bile in the upper small intestine of most of these dogs. But, for all that, the gall bladder was never found empty; it was usually about half full. It would seem that the secretion of bile must have been accelerated.

The colon and rectum were affected in much the same way as the small intestine, except that the inflammatory reddening was chiefly along the summits of the longitudinal ridges. The colitis was recorded as hemorrhagic in 1, severe catarrhal in 3, moderate in 28, slight in 2, absent in 1, and was not recorded in 11. Microscopically, the three colons examined showed a marked exudation of mucus in addition to the hyperemia and lymphocytic infiltration similar to that observed in the small intestine.

One other lesion appeared to be of outstanding diagnostic importance, namely, a very intense hyperemia or congestion of the kidneys. In 38 cases (82%), the change was classified as severe or marked, which means that grossly the kidneys (both right and left) were a deep blood-red color. In no other disease has the writer encountered such severe engorgement with blood as many of these kidneys showed. Kidneys of the remaining animals were listed either as slightly hyperemic or as spotted. The latter condition was a mottling of red and gray, the red being due to an excess of blood, the gray to degenerative changes. In some, the medullary rays were visible as radiating white streaks because of fatty changes in them.

Microscopically (7 cases were examined), the engorgement with blood involved capillaries in all parts of the kidney and the veins. It probably represented both active hyperemia of inflammatory inception and the passive congestion of a terminally waning circulation. The condition was most severe in the medulla and renal pyramids, where actual hemorrhage took place in a few kidneys. There were also degenerative changes of limited extent: cloudy swelling of epithelium of some convoluted tubules, and lipoidosis of the tubular epithelium in many medullary rays. One individual showed a chronic inflammatory reaction, but this undoubtedly antedated the acute illness which results from this poison.

The preceding are the postmortem lesions which were found to be of diagnostic significance. Certain others, while not pathognomic, are deserving of mention. The liver was usually discolored in the direction of pale yellow, but mottled with a reddish hue. Microscopically (5 livers were examined), this was attributable to an excess of blood in the form of passive congestion, which was present in all cases, together with a variable amount of toxic degeneration (toxic hepatitis). Central necrosis, cloudy swelling throughout the lobule, and occasional lymphocytes in the islands of Glisson (portal spaces) were the toxic changes encountered. Stainable fat was practically absent when the tissues were submitted to the microscope, although at the autopsy table it had been repeatedly suspected because of the discoloration of the liver.

The spleen was invariably small and usually empty of blood. The three spleens examined microscopically had very atrophic pulp. One showed lymphoid hyperplasia, doubtless the result of some pre-existing infection.

Cystitis, characterized grossly by reddening of the mucosa and microscopically (3 examined) by hyperemia and superficial desquamation, was noted in several cases. In 5, the bladder was full at time of necropsy; in the remainder, records are lacking on this point.

The cranial cavities were opened in 4 cases (3 natural, 1 experimental). All showed an excess of venous and capillary blood, doubtless passive congestion, in the pia-arachnoid and in the cerebral parenchyma. Microscopic sections of the brain were not made.

SUMMARY

The important postmortem lesions of poisoning by alpha-naphthyl thiourea in dogs

are: (1) hydrothorax found to some extent at least in 100 per cent of cases; (2) severe pulmonary edema in 98 per cent; (3) catarrhal gastroenteritis in 95 per cent; (4) unusual amounts of bile in the intestine found in a high but not accurately determined percentage of cases; and (5) very severe renal hyperemia well marked in 82 per cent and existing to some degree in 100 per cent of cases examined.

The final diagnoses of the 46 cases from which these conclusions are derived were based upon: (1) a history of experimental administration in 16 instances;* (2) the practical certainty or strong suspicion of the owner that the dog had eaten the poison in many instances; (3) the clinical symptoms, which are believed to be typical; and (4) the fact that this syndrome of lesions made its appearance concurrently with the accessibility of the poison in a campaign to exterminate rats, occurred in dogs known to have been poisoned, and has never been known previously from any other cause.

Part 3†

EXPERIMENTAL OBSERVATIONS ON TOXICITY

In view of the deaths occurring among the dogs in the community following the rat extermination campaign, it seemed desirable to study the action of this rodenticide upon dogs.

Richter[1] has reported that ANTU would kill dogs under some circumstances, but that dogs were usually protected by the emetic properties of the drug. Dieke and Richter[2] found that 20 mg. per kg. of body weight was insufficient to kill any of 7 mature dogs, whereas, 50 mg. per kg. killed all of them. They also noted that 3 puppies were more resistant to ANTU than adult dogs. The importance of age in the susceptibility to ANTU has been more clearly defined in the case of the common wild rat than in any other animal. Suckling rats were found to be about 7 times as resistant as the mature rats and the susceptibility of the intermediate ages was proportional.[3] Chanutin *et al.*[4] developed a tolerance in 2 adult dogs to the acute toxic effects of ANTU by increasing the oral dosage from 10 mg. per kg. to 150 mg. per kg. of body weight within twenty days.

Fatal Doses of ANTU.—ANTU was administered orally to 17 dogs (table 1). It was determined that ANTU could be administered orally with the same effect whether given by capsule, in a 10 per cent acacia suspension in water, or in ground horse meat. The dogs were secured from a variety of sources and generally were of mixed breedings.

The data in table 1 indicate that mature and old dogs were considerably more susceptible to the toxic effects of ANTU than were young dogs. These data confirm the findings of Dieke and Richter.[2] Dogs 1 to 7, inclusive, were immature and survived dosages of ANTU up to almost three times the fatal dose of 30 mg./kg. for mature dogs. No effort was made to calculate the m.l.d. because this undoubtedly varied with the ages of the dogs; and the number of animals was insufficient to justify any exact conclusions. However, the data indicated that for dogs around 5 to 10 months of age, the fatal dose of ANTU was between 85 and 100 mg./kg. of body weight. For dogs from 2 to 14 years of age, the fatal dose was probably around 30 to 50 mg./kg. of body weight, although this was less certain.

A sample of ANTU was recrystallized

TABLE I—The Effects on Dogs of a Single Oral Dose of ANTU

Dog (No.)	Age	Dosage (mg./kg.)	Vomition	Dyspnea	Outcome	Gastro-enteritis	Hydro-thorax	Pulmonary edema
1	10 mo.	10	Ab.	N.	Rec.			
2	10 mo.	20	Ab.	N.	Rec.			
3	10 mo.	30	Ab.	N.	Rec.			
4	10 mo.	40	Pres.	N.	Rec.			
5	10 mo.	50	Pres.	N.	Rec.			
6	6 mo.	75	Pres.	N.	Rec.			
7	5½ mo.	85	Ab.	N.	Rec.			
8	8 mo.	100	Ab.	N.	D. 14 hr.	Mod.	Mod.	Pres.
9	7½ mo.	115	Pres.	N.	D. 30 hr.	Mod.	Mod.	Pres.
10	10 mo.	130	Ab.	N.	D. 14 hr.	Mild	Mod.	Pres.
11	10 mo.	125*	Ab.	N.	D. 4 hr.	Severe	Mod.	Pres.
12	Old	150	Pres.	Ext.	D. 15 hr.	Mod.	Mod.	Pres.
13	Mature	75	Ab.	Mod.	D. 30-42 hr.	Mod.	Exc.	Pres.
14	2 yr.	75	Ab.	Ext.	D. 12-24 hr.	Mild	Exc.	Pres.
15	4 yr.	50	Pres.	Mod.	D. 18-20 hr.	Severe	Exc.	Pres.
16	12 yr.	30	Ab.	Ext.	D. 12 hr.	Mod.	Exc.	Pres.
17	14 yr.	30	Pres.	Ext.	D. 16-18 hr.	Mod.	Exc.	Pres.

*ANTU purified by recrystallization from absolute ethanol.
Ab. = Absent; Pres. = Present; N. = None; Ext. = Extreme; Mod. = Moderate; Rec. = Recovered. D. = Died in; Exc. = Excessive.

*These constituted 16 of the 17 experimental dogs reported in Part 3 of this paper.

†Part 3 was prepared by Dr. L. Meyer Jones. The assistance of the staff of the Stange Memorial Clinic in providing facilities and help in observation of the experimental animals is appreciated.

from absolute ethanol to remove any toxic impurity that might be present. Dog 11 received 125 mg./kg. of the recrystallized drug and died within four hours with a very acute but typical case of ANTU poisoning. This period of four hours was three to seven times shorter than the usual course of a poisoned dog. The impurity removed from the drug by recrystallization represented about 16 per cent by weight of the total crude drug.

Development of Tolerance.—Five Collie dogs, 10 months of age, litter mates, and raised in the same kennels, were dosed orally with ANTU at five successive times. Table 2 shows the dosages administered. Four dogs developed a tolerance to ANTU of approximately ten times the original oral dose within about three weeks' time and after a total of five administrations of drug. Dog 2 succumbed after the third dose which consisted of 300 mg./kg. of body weight.

Table 2 shows that the dogs used in this experiment repeatedly received amounts of ANTU in excess of the initial fatal dose of 85 to 100 mg./kg. This clearly demonstrated that the dogs developed a tolerance to repeated doses of ANTU.

A tolerance to ANTU in the vomition reflex also developed. During the first administration, vomition occurred in the dogs receiving 40 mg. or more per kilogram of body weight. Vomition did not occur at the 50 to 100-mg./kg. level on the fourth administration of drug, presumably because of the intervening high dosages.

Parenteral Administration.—Five immature dogs which survived orally administered ANTU received more drug by parenteral injection.

TABLE 2—The Development of Tolerance in 10-Month-Old Dogs to Oral Doses of ANTU

Date	Dog (No.)	Dosage (mg./kg.)	Vomition
11- 8	1‡	10	Ab.*
	2§	20	Ab.
	3§	30	Ab.
	4‡	40	Pres.**
	5‡	50	Pres.
11-13	1	75	Pres.
	2	100	Pres.
	3	125	Pres.
	4	150	Pres.
	5	175	Pres.
11-14	1	75	Pres.
	2†	300	Pres.
	3	125	Pres.
	4	450	Pres.
	5	175	Pres.
11-20	1	25	Ab.
	3	50	Ab.
	4	75	Ab.
	5	100	Ab.
12- 2	1	200	Pres.
	3	300	Pres.
	4	400	Pres.
	5	500	Pres.

*Ab. = Absent. **Pres. = Present.
†Died in 34-36 hr. showing extreme dyspnea and cyanosis. Autopsy revealed a mild gastroenteritis, excessive hydrothroax, and pulmonary edema.
‡Female. §Male.

The data in table 3 on dog 1 suggests that a tolerance to the intravenous injection of ANTU may be developed. After an initial dose of 0.6 Gm. proved to be inadequate, a dose of 1 Gm. injected on the following day was not fatal despite this amount being fatal to litter mates 4 and 5. A total of 2 Gm. administered intravenously within seven minutes was necessary to produce fatal depression of the respiration.

The dogs receiving a fatal dose of ANTU intravenously died within seven to ten minutes as a result of respiratory failure. This rapid action of ANTU suggests that the inhibition of respiration was due to its action upon the central nervous system.

Vomition was observed in only 1 dog receiving ANTU intravenously. Vomition became more or less continuous in the dog which received ANTU by intraperitoneal injection. This animal was extremely dehydrated at its death, as well as severely depressed and weakened.

DISCUSSION

In view of the large number of dogs that died during the local rat extermination campaign, it seemed desirable to emphasize the toxicity of ANTU in the dog. This view was not entirely in agreement with the original statements by Richter,[1] who claimed that "the emetic properties of ANTU protected dogs in most cases." However, during this study, clinical observations on experimental dogs and clinical patients indicated that the extent to which the stomach was filled with ingesta may have an influence on the chances of the dog for recovery. The original experiments[1] for testing the toxicity of ANTU were performed on dogs that were starved over night. Obviously, their stomachs were empty when the ANTU was administered, and the entire gastric mucosa was exposed to the irritant action of the drug. It was little wonder that most of the dogs vomited the drug. However, the situation appeared to be different in the dog that received ANTU into a full or partly full stomach. In this case, the ingesta undoubtedly protected the gastric mucosa to some extent from the irritative action of ANTU. Thus, emesis did not occur or was slow to occur, so that some of the ANTU was carried into the small intestine. The alkaline secretions of the intestine increased the solubility and absorbability of ANTU with a consequently greater toxicity.

The following clinical observations made on experimental dogs supported the aforementioned point of view. The dogs that

received lethal doses of ANTU after starving vomited promptly. The dogs which had been fed shortly before receiving ANTU either did not vomit at all or not until visible signs of ANTU toxicity were present. These observations on experimental dogs were corroborated by clients who described the behavior of their own poisoned dogs which were brought to the veterinary clinic for treatment. The usual history included the statement that the dog had been confined except after eating, when it had been released for brief exercise. There was no way of knowing how many dogs ingested ANTU bait on an empty stomach and vomited the drug.

It seemed unwise to attempt a rather exact calculation of the m.l.d. for ANTU in dogs because of the apparent variation in susceptibility according to age. Many dogs would be needed for this study. Furthermore, the correlation between susceptibility and age has been well worked out on rats by Dieke and Richter.[2]

The dogs dosed orally with ANTU all showed varying degrees of gastroenteritis. This local irritation undoubtedly promoted vomition. How important a factor the gastroenteritis may have been in causing vomition was difficult to determine because dog 1 vomited following the intravenous injection of ANTU. Thus, it would appear that ANTU also stimulated the emetic center of the brain.

Fatal respiratory embarrassment was observed in all dogs receiving ANTU. At autopsy, the lungs were edematous in each case and the thoracic cavity contained from moderate to excessive amounts of serous fluid. The dogs dying from ANTU administered intravenously showed slight to moderate pulmonary edema but no hydrothorax. For a complete discussion of the pathology of the experimental animals, the reader is referred to Part 2 of this paper.

TABLE 3—Some Effects of Parenterally Administered ANTU upon Dogs Rendered Tolerant by Previous Oral Ingestion of ANTU

Date	Dog (No.)	How Admin.	Amt. of ANTU	Clinical Effect	Autopsy
12-6	1	I.V.	0.6 Gm.	Depression of respiration, vomition, recovery. No prominent effect.	
12-7	1	I.V.	1.0, 0.5, 0.5 within 7 min.	Vomition, depression of respiration, cyanosis, collapse, inhibition of respiration.	Lungs edematous, no hydrothorax.
12-6	4	I.V.	1.0	Inhibition of respiration.	Slight edema and congestion of lungs, no hydrothorax.
12-6	5	I.V.	1.0	Same.	Same.
12-6	6	I.V.	1.6	Same.	Same.
12-6	3	I.P.	2.0	Intermittent but persistent vomition; no prominent respiratory distress; death in 24 hours.	Slight hydrothorax, no gross pulmonary edema, free ANTU at site of injection.

The mechanism of action whereby ANTU produced death was most interesting and unusual. Death in the dog and the Norway rat was the result of a drowning pulmonary edema. The ANTU greatly increased the permeability of the lung capillaries, until the quantity of tissue lymph formed was beyond the collecting capacity of the lymph vessels. Drinker[4] has shown that ANTU may increase the flow of lymph from the lungs by 80 times. Thus, when the lymphatic drainage from the lungs became inadequate, the lung tissue became waterlogged with a resultant decrease in its ventilating capacity. The only route for escape of the excess fluid was through the air passages which progressively excluded air and led to death. Why or how ANTU influenced the permeability of the lung capillaries is not understood. Neither is it clear why ANTU affected only the vessels of the lung and not of other organs.

CONCLUSIONS

1) Mature and old dogs were considerably more susceptible to the toxic effects of ANTU than were immature dogs. The fatal dose of ANTU for dogs around 5 to 10 months of age was between 85 and 100 mg./kg. of body weight. The fatal dose for dogs from 2 to 14 years of age approached 30 mg./kg. of body weight.

2) A tolerance to the acute poisonous effects of ANTU was readily developed in immature dogs by oral administration of the drug.

3) Dogs rendered tolerant by previous oral ingestion of ANTU were susceptible to ANTU injected intravenously or intraperitoneally.

4) Pulmonary edema and excessive hydrothorax were the immediate causes of death in the dogs poisoned orally with ANTU.

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Part 4*

THE IDENTIFICATION OF ALPHA-NAPHTHYL THIOUREA (ANTU) IN BIOLOGICAL MATERIALS FROM DOGS

A chemical test for alpha-naphthyl thiourea (ANTU) dispersed in biological material such as ingesta or vomitus would be of value in the identification of ANTU as the cause of death in a pet or, under exceptional circumstances, as an aid in diagnosing the sickness of a patient.

Advice from chemists[1] indicated that there was an established chemical test devised by Grote[2] that might be satisfactory for identifying this type of compound. This test depended upon the presence of the C=S linkage to produce a green to blue color which is a positive reaction. The test, then, would be specific for thiourea compounds and not for alpha-naphthyl thiourea (ANTU) alone. However, it is rather unlikely that a thiourea-type compound would often be present in biological material from carnivorous animals to confuse the identification of ANTU. A simple but rather long procedure for the isolation of ANTU from viscera was reported by Dybing.[3] The identification was based upon the same color reaction as was described above. Eighty different drugs that might interfere with the reactions were tested and all gave negative color reactions. A clinical report[4] of 9 dogs, in which 8 fatalities occurred, also emphasized the toxicity of ANTU. The presence of pulmonary edema and hydrothorax were confirmed by roentgenograms. The electrocardiogram was thought to be characteristic.

MATERIALS AND METHODS

The thiourea test reagent of Grote is easily prepared, but it deteriorates rapidly, even at refrigerator temperature. It should be prepared fresh for each occasion or, at least, stored in a refrigerator for no more than two to three days before use. The directions for preparation of the test solution are as follows:

Dissolve 0.5 Gm. sodium nitroprusside in 10 cc. of water at room temperature.

Add 0.5 Gm. hydroxylamine hydrochloride.

Add 1.0 Gm. sodium bicarbonate, after the evolution of gas ceases.

Add 2 drops of liquid bromine; an excess ruins the color reactions.

Remove excess bromine by aëration of the solution where poisonous fumes may escape harmlessly.

Filter and dilute with water to a total volume of 25 cc.

A search for a solvent to extract ANTU from biological mixtures indicated that chloroform was the most suitable of a wide range of organic solvents. ANTU is insoluble in water but will dissolve in chloroform. A biological sample, vomitus or urine for example, would be collected in a container to which chloroform could be added to dissolve the ANTU. The mixture should be shaken at intervals for a period of several hours. After setting for a day or more, the chloroform extract should next be filtered into an evaporation dish and the chloroform allowed to volatilize. The residue should next be dissolved in a few cubic centimeters of acetone which renders the sample ready for testing with the thiourea test reagent. The use of acetone as a final solvent is rather important because a solvent is needed which will dissolve both the fat-soluble ANTU and the water-soluble thiourea test reagents. The most favorable proportions for testing were found to be about 2 cc. of acetone solution of the unknown, plus 2 cc. of distilled water, plus 0.5 cc. of fresh thiourea test reagent, plus an excess of sodium bicarbonate. Table 1 indicates the color responses.

Samples were collected from dogs experimentally dosed with ANTU and tested with

TABLE I—The Sensitivity of Fresh Thiourea Test Reagent for Alpha-Naphthyl Thiourea (ANTU)

ANTU in mg.	cc. of reagent	Color by reflected light					
		5 min.	15 min.	30 min.	45 min.	60 min.	90 min.
2.000	0.5	Deep blue	Same	Same	Same	Same	Faded
1.000	0.5	Light blue	Same	Same	Same	Same	Faded
0.800	0.5	Light blue	Same	Same	Same	Same	Faded
0.600	0.5	Light blue	Same	Same	Same	Same	Faded
0.400	0.5	Light blue	Same	Same	Same	Same	Faded
0.200	0.5	Greenish blue	Same	Same	Same	Same	Faded
0.100	0.4	Light green	Same	Same	Same	Faded	Faded
0.050	0.4	Light green	Same	Same	Fading	Faded	Faded
0.025	0.2	Yellowish green	Same	Same	Fading	Faded	Straw yellow
0.013	0.2	Greenish yellow	Same	Same	Fading	Straw yellow	Straw yellow
0.000	0.2	Straw yellow	Same	Same	Straw yellow	Straw yellow	Straw yellow

*Part 4 was prepared by Dr. L. Meyer Jones.

fresh thiourea test reagent. The results are listed in table 2.

TABLE 2—Results Using the Thiourea Test Reagent for Testing Samples from Dogs Dosed with ANTU

Dog (No.)	Sample	Test Color
1	vomitus	deep blue
2	urine	light blue
3	vomitus	deep blue
4	vomitus	deep blue
5	vomitus	deep blue
6	urine	light green
	vomitus	deep blue
	bile	(light green-?)
	pleural effusion	greenish yellow
	blood	negative
7	ingesta	deep blue
8	vomitus	deep blue
9	ingesta	deep blue
	pleural effusion	negative
10	ingesta	deep blue
	bile	(light green-?)
11	ingesta	deep blue
	pleural effusion	greenish yellow
12	ingesta	deep blue
	pleural effusion	negative

Samples were obtained for analysis from a few of the clinical cases that were brought to the Iowa State College Veterinary Clinic. ANTU poisoning was diagnosed in all of these dogs as a result of clinical symptoms and postmortem examination.

To further substantiate the above data, samples of ingesta were collected from the stomach and small intestine of each of 5 canine patients in the clinic that died of other causes. These samples all gave negative color tests with the thiourea test reagent.

The findings in table 3 correlate with the clinical and autopsy findings in all cases except one. In the latter, case 47907, an undesirable amount of pigment was extracted by the chloroform and dissolved by acetone so as to interfere with the test. This interference of pigments illustrates one of the limitations of the thiourea-reagent test. Bile pigments particularly are carried by the solvent used so that they largely mask any positive color reaction that may occur. This interference was illustrated in dogs 6 and 10 listed in table 2.

The identification of ANTU in the urine indicates that ANTU is excreted by the kidneys. In these cases of acute poisoning, no feces were passed after the ANTU was administered to permit testing for excreted or unabsorbed ANTU. The occurrence of three faintly positive tests among seven samples of pleural effusion fluid would indicate that ANTU is present in the tissue fluid of the lungs. ANTU undoubtedly is present in blood despite the fact that one sample of blood tested negative for a thiourea type compound.

TABLE 3—A Correlation of Thiourea Test Reagent Results with Positive Clinical Diagnoses

Case (No.)	Sample from	Test Color
47889	stomach	light green
	intestine	deep blue
47892	stomach	light green
	intestine	(pigment interference)
47907	stomach	negative
	intestine	(pigment interference)
48072	stomach	light green
48080	stomach	light green
48100	intestine	deep blue
	pleural effusion	yellowish green
48107	intestine	deep blue
	blood	negative
48156	intestine	deep blue
	pleural effusion	negative
48716	intestine	deep blue
	pleural effusion	negative

DISCUSSION

The use of the thiourea test reagent of Grote to identify ANTU in biological samples has several disadvantages. The test is not specific for ANTU; it will identify the thiourea type of compound but not ANTU alone. The reagent is unstable, even at refrigerator temperatures, and should be freshly prepared for use. Bromine, one of the ingredients, is somewhat dangerous to handle as well as to store. There may be considerable interference in the color test from pigments which are extracted by the solvents along with the ANTU. No doubt, some of this could be avoided by further purification before testing. The thiourea test at present is qualitative in nature, although within narrow limits there is a variation in color that is roughly proportional to the concentration.

The thiourea test reagent can be used to analyze samples from both living and dead animals, although its use as a diagnostic aid in fatalities would undoubtedly predominate. However, samples of vomitus or enteral washings or urine from the living patient, as well as suspected feed, could be analyzed by the veterinarian for ANTU content.

CONCLUSIONS

The use of the thiourea test reagent of Grote is suggested for the probable identification of alpha-naphthyl thiourea (ANTU) in biological materials collected antemortem or postmortem from dogs. A positive test would indicate strongly that ANTU is present because of the improbability of finding other thiourea-type compounds. Limited data are reported to support use of the test. The test reagent has some inherent disadvantages which limit its use.

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The Electrocardiogram of the Chicken

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NO STUDIES ON electrocardiography of the chicken have been made in recent years. Earlier studies upon the normal electrocardiogram (ECG) and rate of conduction in the bird heart were made by Mangold and Kato,[1] Kahn,[2] Firket,[3] Lewis,[4] and others. These investigators showed that the normal ECG of the bird was different from that in man and certain other mammals, but they studied only a few birds, in many cases 5 or fewer. With such small numbers of specimens, it was impossible to study the variations in the bird ECG or to determine what constitutes the normal or abnormal picture.

It is conceded by most poultry pathologists that a large part of the adult mortality in chickens results from nonspecific diseases or disorders (*see* Hutt,[5] Lubbehusen and Beach,[6] Johnson,[7] and others) It appeared possible that some of these deaths are caused by heart disorders, and that some of them might be revealed by the electrocardiogram.

To investigate this possibility, studies were made on serial electrocardiograms of adult birds, and the variations in heart rate, intervals of time, amplitude, and electrical axes of the ECG's were recorded and analyzed.

MATERIALS AND METHODS

From three to six serial electrocardiograms per bird were taken on 72 White Leghorn females over the course of a year. The first ECG's were taken at 5 to 6 months of age.

Contrary to the practice of previous workers, the birds used in this study were not anesthetized before the ECG's were taken. The birds were strapped into a V-shaped holder, with a hole in the trough, through which the legs extended. The legs were tied to the back of the holder to prevent movement.

Needle electrodes were inserted in the muscles at the base of right and left wings, and the muscle of the left thigh, to form the standard limb leads. Thus, lead I ran from right wing to left wing; lead II from right wing to left leg, and lead III, from left wing to left leg. The bird was then covered with a cloth and allowed to quiet down before the electrocardiogram was taken.

The ECG's were recorded with a direct-writing, moving-coil galvanometer (cardiotron) manufactured by the Electro-Physical Laboratories, New York, N. Y. This instrument, according to the manufacturers, has a deflection speed of 0.007 second per centimeter. Rappaport and Rappaport[8] showed that with animals having heart rates of 750 to 1,000 per minute, a deflection speed of 0.0015 is necessary to prevent notching and distortion in the tracings. Since there was little or no distortion observed in the ECG's of this study, the cardiotron appears fast enough for the chicken heart. Lewis[4] used an electrocardiograph with standard speed in his work with the chicken. All ECG's were standardized at 1 cm. = 1 millivolt, with a chart speed of 25 mm. per second, unless stated otherwise.

RESULTS

The Normal Electrocardiogram of the Chicken.—The auricular wave of depolarization is the P wave. There is no recognizable wave of repolarization. The ventricular waves of depolarization are the R and S waves. There is no Q wave. T is the wave of ventricular repolarization. In leads II and III, R is usually small or absent but usually is more prominent in II; S is prominent and T is upright. P, when present, is always upright in II and in most cases in III (fig. 1). It may be absent or fused with the T waves in some cases, particularly when the heart rate is rapid (fig. 2). The electrocardiogram, according to Lewis,[4] records preponderantly electrical activity of the left ventricle; in other words, it is mainly a levocardiogram.

TABLE I—Configuration of R and S in Lead I

Type of I	Birds (No.)	Per cent	ECG's (No.)	Per cent
A	13	18.05	27	13.17
B	46	62.90	129	62.70
A + B	13	18.05	49	23.90
Totals	72		205	

Type A = prominent S, with small or absent R.
Type B = prominent R, with small or absent S.
A & B = R and S of about same amplitude.

The ECG of lead I of the normal bird is the most variable of the limb leads. In most cases, the amplitude of all waves is very low, or some of the waves may be isoelectric.

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The ECG of lead I exhibits several types of configurations with respect to R, S, and T.

With respect to R and S there are two main types:

Type A is characterized by an upright P, followed by a relatively prominent S wave. The T wave may be upright, flat, isoelectric, or slightly negative (fig. 3A). Eighteen per cent of the birds studied showed lead I's of this type (table 1).

Type B is characterized by an upright P, followed by a relatively prominent R, and no S, or a small S. The T wave is usually isoelectric or slightly negative but may be slightly positive (fig. 3B). Approximately 63 per cent of the birds studied exhibited this type (table 1).

Eighteen per cent of the birds studied showed lead I's, in which the R's and S's were of about equal prominence.

Abnormal and Unusual ECG's.—Table 2 presents a summary of the number of birds studied and the mortality and percentage of birds exhibiting abnormal or unusual electrocardiograms. Among the 20 birds which died during the year (27.7%), only 3 (15%) had abnormal or unusual ECG's, as described in the table. The ECG of one of the birds showed an alternation of amplitude in leads II and III (fig. 4, bird 5). This bird died shortly after the ECG was taken. Most of the deaths of the birds were due to coryza and nonspecific disorders.

Of the 52 birds which survived the test period (birds at least 18 months old), 28.8 per cent revealed abnormal or unusual ECG's (table 2). Five of these birds (9.6%) exhibited transient abnormalities of the ECG. Four of them showed flattened or inverted T waves; subsequently, the ECG's were normal (fig. 5, bird 4B). Bird 7 (fig. 4) exhibited alternated rhythm and amplitude. This change was also transient.

The ECG's of 7 birds showed consistently a more prominent R II and R III than S II

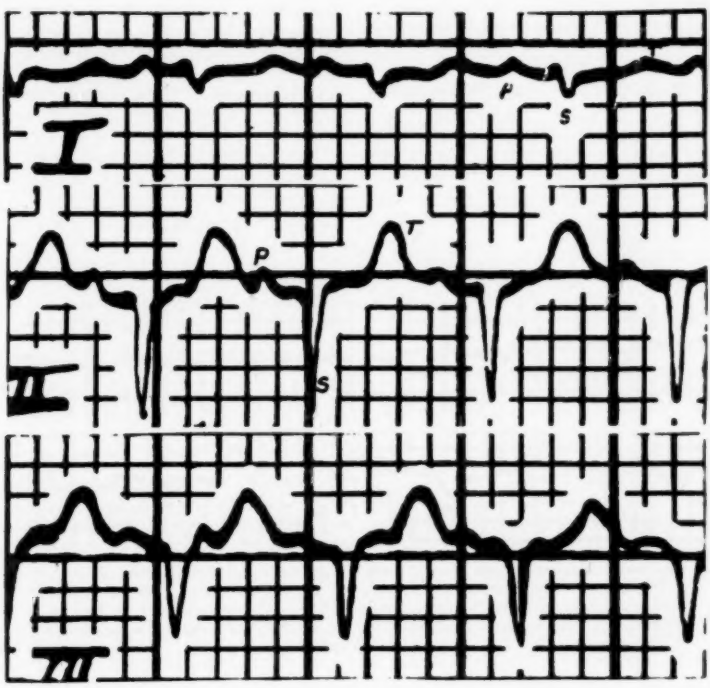

Fig. 1—Leads I, II, and III of a normal electrocardiogram.

The electrocardiograms were photographed as follows: The original tracings were black lines on white background. These were photographed with no changes. Some of the black time and amplitude lines around the actual ECG were then blocked out with white paint from the print by the photographer and then rephotographed. This gives more contrast. The actual tracing was not changed.

and S III (fig. 6), and the amplitude was of low order. Three birds showed unusually low amplitude in all leads and all ECG's.

Intervals of the Chicken Electrocardiogram.—Intervals of P, S, T, P to S, and from the beginning of S to the end of T were determined for leads II and III. The

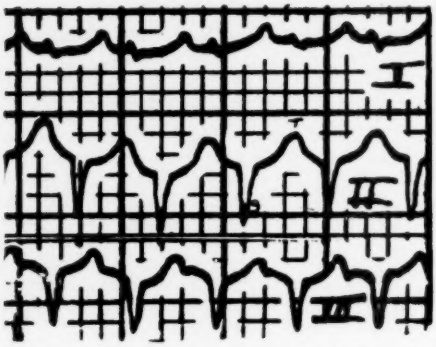

Fig. 2—Leads I, II, and III of a normal electrocardiogram where the P wave is fused with the T wave.

R wave in II and III is usually small or absent, and when present, its starting point is difficult to locate. For that reason, the P-R interval was not determined. In most cases, there is no ST segment; T usually begins where S ends, and that point was used as the starting point for T. Measurements were made only on those ECG's in which the various waves were clear cut. These were made on 41 birds, but in some cases include more than one ECG per bird. The heart rates varied from 180 to 340 per minute with a mean of 279. Only 3 birds had rates less than 220 per minute. The heart rates of the different birds and ECG's were grouped arbitrarily according to rate, and the influence of the rate on the intervals was determined. The results are shown in table 3. In general, the interval for P decreased as heart rate increased; however, the differences in intervals for some of the groups were not significant, as might be expected from arbitrary groupings.

The mean values for S varied from 0.0242 second to 0.0233 second for the different groups, but none of these differences were significant, nor did the heart rate influence the S intervals. Lewis,[4] who determined the S interval on 5 anesthetized chickens, reported values ranging from 0.0279 second (heart rate of 210) to 0.0378 second. The heart rates for all except 1 were below 165 per minute.

The T values ranged from 0.1161 to 0.0925 second and varied inversely with the heart rate. Not all of the group differences, however, were significant.

The interval from P to S (P-S) ranged from 0.0812 to 0.0723 second, and most of the group differences were significant.

The interval from the beginning of S to the end of T (ST) ranged from 0.1402 to 0.1164 second. Thus, as the heart rate increased, the intervals for all except S decreased.

Amplitudes.—A discussion of the amplitudes of waves in leads I, II, and III follows.

Amplitude in Lead I.—The amplitude of all waves in lead I is very low. On many of the ECG's, the thickness of the baseline in relation to the amplitude is such that it is difficult or impossible to get an accurate measurement with the usual standardization of 1 cm. = 1 millivolt. Therefore, direct measurements can be made only on those ECG's which show appreciable ampli-

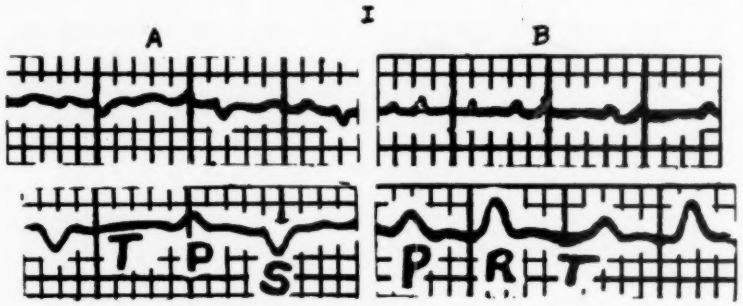

Fig. 3—Electrocardiograms of lead I exhibiting two types of configurations, A and B. The upper tracings were taken at the usual standardization and chart speed. Each lower tracing shows the same configuration as the one above, but the standardization is 1 cm. equals 1.6 millivolt, and the chart speed is approximately 60 mm. per second.

tudes. Values thus obtained are too high and do not represent average amplitudes. For accurate measurements of lead I, the standardization should be increased considerably. This was not done in this study.

A better estimate of average amplitude of the waves in lead I can be deduced from equal to the difference between II and III, which is 0.0181 millivolt. P I normally may vary from almost isoelectric to about 0.04 millivolt.

R.—The mean amplitude for lead II was 0.0346 and for lead III, 0.0021. The difference is significant. These values represent

TABLE 2—Mortality and the Number of Chickens Showing Abnormal or Unusual ECG's

Birds (No.)	Dead (No.)	Dead (%)	Birds dying abnormal ECG's (No.)	Birds dying abnormal ECG's (%)	Birds surviving Abnormal ECG's (No.)	Birds surviving Abnormal ECG's (%)	Birds surviving Unusual ECG's (No.)	Birds surviving Unusual ECG's (%)
72	20	27.7	3*	15	5	9.6	10	19.2

Description of abnormal or unusual ECG's

2* inverted P III 1* alternation of amplitude	died	4 T-wave flat or inverted, transient 1 alternation of rhythm and amplitude 7 prominent R II, usually higher than S II 3 very low amplitude	alive

the differences in the mean amplitudes for the various waves in leads II and III in accordance with Einthoven's law. This is discussed under amplitudes for leads II and III.

Amplitudes in II and III.—The determinations were made on 35 to 41 birds, and in some cases, include values for more than one ECG per bird. Measurements were made on 5 to 10 cycles per ECG per bird and the averages of these taken. The results are shown in table 4.

P.—The amplitude for P in lead II averaged 0.0602 millivolt, and for lead III, 0.0421. The difference, 0.0181, is statistically significant.

In accordance with Einthoven's law, the amplitude of I and III equals II. If P II is greater than P III, then P I should be the means of all lead II's and III's regardless of the configuration of R or S in lead I. When the R II's and III's were grouped and measured according to the type of lead i, the resulting values were virtually the same.

S.—It was found that the amplitude of the S waves and the RS complexes varied in general according to the type of lead I exhibited by a given ECG. For those birds exhibiting a type A lead I (R small or absent, with a relatively prominent S), the amplitudes of the S II's and RS II's were greater than for lead III. Conversely, when in lead I the S was absent and a relatively prominent R was present (type B), the S III's and RS III's were greater than those for lead II.

The mean amplitude for S II (type A)

TABLE 3—Intervals of the Chicken Electrocardiogram in Seconds

	Heart rates	221 240	241 260	261 280	281 300	301 340	T* values (column)
P	Mean	0.0408	0.0429	0.0421	0.0410	0.0374	3 and 5
	Minimal	0.0350	0.0390	0.0400	0.0350	0.0310	4 and 5
	Maximal	0.0480	0.0450	0.0450	0.0470	0.0440	
S	Mean	0.0242	0.0247	0.0234	0.0233	0.0235	
	Minimal	0.0210	0.0200	0.0200	0.0200	0.0200	
	Maximal	0.0260	0.0290	0.0310	0.0270	0.0280	
T	Mean	0.1161	0.1095	0.1048	0.1043	0.0925	2 and 5
	Minimal	0.1230	0.1220	0.1210	0.1180	0.1110	3 and 5
	Maximal	0.1710	0.1590	0.1550	0.1330	0.1250	4 and 5
P-S	Mean	0.0812	0.0847	0.0849	0.0790	0.0723	2 and 4
	Minimal	0.0760	0.0720	0.0760	0.0720	0.0670	3 and 4
	Maximal	0.0900	0.0910	0.0940	0.0860	0.0820	3 and 5 4 and 5
ST	Mean	0.1402	0.1342	0.1281	0.1270	0.1164	2 and 3
	Minimal	0.1230	0.1220	0.1210	0.1180	0.1110	2 and 5
	Maximal	0.1710	0.1590	0.1550	0.1330	0.1250	3 and 5 4 and 5
	No. of ECG's	7—9	9—12	16	14—16	12—14	

*Significant values. All means reliable.

was 0.2193 and for S III (type A), 0.1787 millivolt. The difference is not statistically significant on the 0.05 level, but closely approaches it. Although, as previously mentioned, approximately 18 per cent of all birds exhibited type A lead I, amplitudes were determined on only part of these birds. Approximately 27 per cent of the ECG's on which amplitudes were determined exhibited type A lead I's.

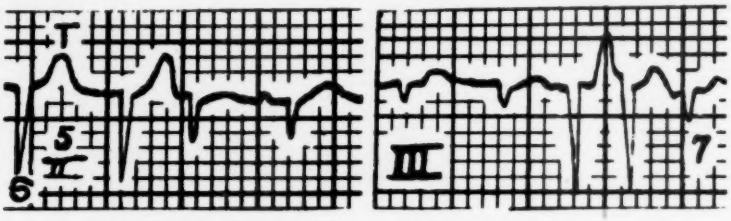

Fig. 4—Electrocardiograms of bird showing alternated amplitude and rhythm.

The mean amplitude for S II (type B) was 0.1241 and for S III (B), 0.1430 millivolt. The difference is not significant.

In many of the type B ECG's, the R wave in lead I was very low or almost isoelectric. In such cases, the difference between the ventricular complexes in II and III decreases. This may account in part for the insignificant differences obtained between S II and S III. The differences between the means of lead II for types A and B, and for lead III of the types are statistically significant. When the two types are grouped as one, the means for S II and S III are 0.1546 and 0.1533 millivolt, respectively.

RS.—RS is equal to the difference between R (positive) and S (negative). These values are used in determining electrical axes, discussed later.

The difference in the means of R and S shown in the table, however, do not equal exactly the values given for RS. This is true because the R values were not grouped according to type of lead I, since they were nearly the same; and also, because the means of R and S values and the RS values were based on different numbers of ECG's in some cases.

The means for RS (type A) leads II and III were 0.1868 and 0.1531 millivolt, respectively. The difference is not statistically significant. The means for RS (B type) leads II and III were 0.0913 and 0.1222 millivolt, respectively, and this difference is significant. The difference between RS (A) and RS (B) lead II was significant, but not that between leads III of the two types. When the types are grouped as one, the means for RS II and III are 0.1195 and 0.1311 millivolt, respectively.

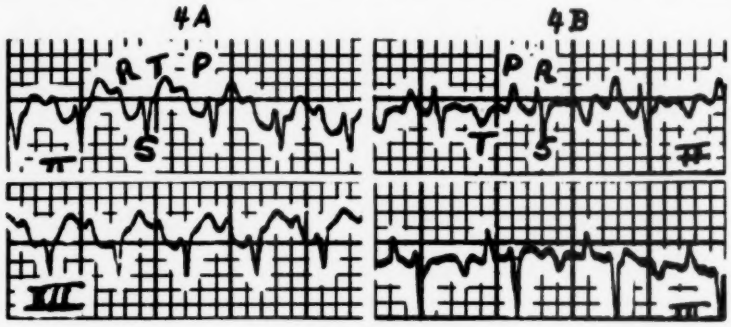

Fig. 5—Leads II and III of a normal ECG (4A) and II and III for the same bird showing an inverted T wave.

R and S in Lead I.—A tenet of Einthoven's law is that the amplitude of any wave or configuration in I and III should equal II. This is true provided all leads are run simultaneously or, if not run simultaneously, there is no change in rate or other changes. In this study, the leads were not run simultaneously, but the agreement between the results obtained and expected appeared to be close. This has been demon-

strated conclusively in another report.[9]

The main ventricular wave of depolarization in II and III is an S, or negative wave, but a small upright R wave is usually present, also. In lead I (type B), however, the main ventricular wave of depolarization is positive, or an R wave. In type A, an R and S may be present but S is more prominent. In order to estimate the amplitude of ventricular depolarization in lead I, it is more accurate to consider the RS's of leads II and III.

Since I and III = II, then I = II — III. Thus RS (A) II (—0.1868) minus RS (A) III (—0.1531) equals —0.0337 millivolt, or RS I. In other words, this figure means that the S complex in I (A) exceeds the R wave by that amount, or that the main ventricular wave is an S. This figure falls within the range of those obtained by the few direct measurements made.

The value for RS (B) II (—0.0913 minus RS (B) III (—0.1222) equals +0.0309 millivolt, or RS (B) I. This value actually represents the amplitude of R I (B), since no S is present in this type of I. This value is within the range of those obtained by direct measurements.

T.—Among the 61 ECG's on which amplitudes were determined, 46 (75%) were instances in which T II was greater than T III. The mean for T II was 0.1095, and for T III 0.0900 millivolt. This difference is significant. In the remaining 15 cases, T II was less than T III. The means for these T II's and T III's were 0.0716 and 0.0901 millivolt, respectively. The difference is significant. If the two categories of T's are grouped, then the means for T II and T III are 0.0933 and 0.0900 millivolt, respectively. This difference is not significant.

That these results are in close agreement with expectations based on Einthoven's law is revealed by the fact that of the 46 ECG's in which T II was greater than T III, 43 (93%) had positive T's in I. Among the 15 cases where T III exceeded T II, 12 (80%) had negative T's in lead I. Of the 6 ECG's which had isoelectric T I's, 3 had T II's which exceeded T III, and 3 had T III's which exceeded the T II.

The amplitude of T I may be estimated from the difference in the means of T II and III. The mean difference for II and III, where II exceeds III, is +0.0195 millivolt (T I positive). The mean difference for II and III, where III exceeds II, is —0.0185 millivolt (T I negative). The estimated values for the T I's agree fairly closely with direct measurements, where such measurements are possible.

Electrical Axes.—Electrical axes may be determined from the records of any two leads. Sturkie[9] has shown that the electrical axes based upon leads I and III or upon leads II and III in the chicken are equally reliable. In this study, leads II and III were used. Values for RS II and RS III were used. Values for RS II and RS III were determined and these values were plotted on the lead II and III arms of Einthoven's triangle. Values for T II and T III were treated in the same manner.

RS Axes.—The axes, like the RS means, were determined according to the type of lead I exhibited by the ECG's. The results are shown in table 5. For ECG's exhibiting type A lead I (RS II greater than RS III), the average electrical axis was —102.11 degrees, with a range of —91 to —120 degrees.

In those cases were RS III exceeded RS II (types B-I), the average electrical axis was —74.04 degrees, with a range of —26

TABLE 4—Amplitudes in Millivolts of P, R, S, RS, and T, in Leads II and III

	ECG's (No.)	II Mean	Stand. error	ECG's (No.)	III Mean	Stand. error	Difference between II and III
P	40	0.0602	0.0026	40	0.0421	0.0016	*
R	56	0.0346	0.0021	56	0.0221	0.0013	*
S (type A)	18	0.2193*	0.0135	18	0.1787*	0.0150	...
S (type B)	43	0.1241*	0.0067	44	0.1430*	0.0071	...
Mean	61	0.1546	0.0125	62	0.1533	0.0070	...
RS (type A)	18	0.1868*	0.0172	18	0.1531	0.0153	...
RS (type B)	43	0.0913*	0.0077	43	0.1222	0.0085	*
Mean	61	0.1195	0.0093	61	0.1311	0.0078	...
T (II > III)	46	0.1095*	0.0056	46	0.0900	0.0050	*
T (II < III)	15	0.0716*	0.0036	15	0.0901	0.0062	*
Mean	61	0.0933	0.0073	61	0.0900	0.0041	...

*Difference statistically significant (T values).

to —103 degrees. All ECG's on which this average is based, except one, ranged below —90 degrees. Three tracings (2 birds) averaged +20 degrees. These birds showed very prominent R waves in leads II and III.

T Axes.—The axes determined were based upon the direction of T in lead I. When T II exceeds III, T I is positive, and

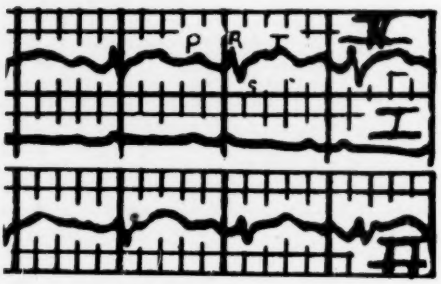

Fig. 6—Electrocardiogram of a bird exhibiting low amplitude and more prominent R II's and R III's than S II's and S III's.

when III is greater than II, T I is negative. When II and III are equal, T I is isoelectric.

The average electrical axis for the ECG's with a positive T I, was +81.8 degrees, with a range of +68 to +89 degrees. For the tracings with a negative T I, the average axis was +100.3 degrees with a range of +95 to 115 degrees. These average axes point to approximately 5 and 7 o'clock, respectively. For the 6 ECG's with isoelectric T I's, the average axis was +89.6 degrees with a range of 88 to 91 degrees. The exact figure to be expected under these conditions is +90 degrees.

DISCUSSION

The results of this study show that heart disorders as revealed by the electrocardiogram accounted for few, if any, deaths of birds during the test period of one year. Of the 3 birds exhibiting apparent abnormalities of the ECG's and which died during the test period, 2 had inverted P III waves. This, in all probability, was not the cause of death. Autopsy revealed nothing specific, and gross observations indicated that their hearts were normal. The inverted P III waves may reflect changes in heart position, since Sturkie[9] has shown that P III, in most cases, becomes inverted when the bird is opened and the heart is exposed.

The 2 cases of alternated amplitude are of interest. One of the birds showing this condition died shortly afterward. The other bird, which survived the test period, is still alive at 2½ years of age. This condition, however, was transient, as previous and subsequent ECG's were normal. Recently, an ECG taken on a pullet afflicted with blue comb disease showed alternated amplitude, characterized by a 2 to 2 alternation of high and low amplitude. The genesis and significance of this condition have not been determined.

The ECG's of 7 of the birds studied (nearly 10%) had consistently more prominent R II's and III's than S II's and III's. The RS electrical axes of these birds ranged from +10 to +30 degrees, whereas the axes of the remaining birds averaged —74.04 and —102.11 degrees, depending on the type of lead I. This suggests that the paths of conduction of the electrical impulse in these birds are considerably different from those of most of the birds studied.

The effects of specific diseases, other than coryza and blue comb, on the electrocardiogram have not been studied.

A number of ECG's have been taken on baby chicks and growing birds. All of these appeared normal and not unlike those of adult birds, although intervals and amplitudes have not been determined.

SUMMARY

Three to six electrocardiograms per bird were run on 72 White Leghorn females over the period of a year. Variations in heart rate, intervals of time, amplitude, and electrical axes were studied.

The results show that heart disorders, as revealed by the electrocardiogram, ac-

TABLE 5—Electrical Axes of Chicken Hearts in Relationship to Type of Lead I

Type of ECG in I	Birds (No.)	ECG's (No.)	ECG's (%)	RS axes: Range axes Degrees	RS axes: Average axes
A	15	17	25.8	−91 to −120	−102.11
B	30 2	46 3	74.2	−26 to −103* +10 to + 30	− 74.04

*Only one ECG above −90 degrees.

Direction of T wave in I	Birds (No.)	ECG's (No.)	ECG's (%)	T axes: Range axes Degrees	T axes: Average axes
Positive	34	43	70.49	+68 to + 89	+ 81.8
Negative	10	12	19.67	+95 to +115	+100.3
Isoelectric	6	6	9.83	+88 to + 91	+ 89.6

counted for few, if any, deaths of birds during the test period of one year.

Fifteen per cent of the birds dying during the period exhibited abnormal or unusual ECG's. Death of only 1 of these birds was thought to be due to the abnormality, and that is questionable.

Approximately 29 per cent of the birds surviving the test period exhibited abnormal or unusual ECG's. These are described and discussed.

The auricular wave of depolarization of the normal ECG is the P wave. The ventricular waves of depolarization are the R and S waves. There is no Q wave. T is the wave of ventricular repolarization.

In leads II and III, R is usually small or absent, but is usually more prominent in II; S is prominent and T is upright. P, when present, is always upright in II and in most cases in III. It may be absent or fused with T in some cases.

The ECG of lead I of the normal bird is the most variable of the limb leads. Lead I exhibits several types of configurations with respect to R and S, but there are two main types, A and B, which are described. Eighteen per cent of the birds studied exhibited leads I of type A, and 63 per cent showed type B. About 18 per cent showed leads I in which the R's and S's were of about equal prominence.

For ECG's showing type A lead I (RS II greater than RS III), the average RS electrical axis was —102.11 degrees; and for those showing type B lead I, the average axis was —74.04 degrees. The average T axis for the ECG's with a positive T I (T II exceeds T III) was +81.8 degrees. For the tracings with a negative T I (T III exceeds T II), the average axis was +100.3 degrees.

The heart rates varied from 180 to 340, with a mean of 279.

The intervals for all of the waves determined, except S, varied inversely with the heart rate.

The intervals and amplitudes are presented and discussed.

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Vaccination Against Newcastle Disease with Formalin-Inactivated, Commercially Produced Vaccines

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SINCE NEWCASTLE disease was first definitely diagnosed in the United States early in 1944 by identification of the causative virus, the problem of its control has been foremost in the minds of poultry pathologists and livestock sanitary officials, not to mention the poultry growers themselves, who have urgently prodded for such control.

Naturally, vaccination with a suitable agent for immunization was among the earliest considerations, as the disease had become so widespread that its control by the slaughter method was impracticable and economically not warranted.[1] In several countries where the disease is much more virulent than in the United States, a modified live virus vaccine has been in use for a number of years. Its experimental use in this country is quite recent. In the United States, field trials with a formalin-inactivated virus vaccine, with or without the incorporation of adjuvants, have been made. At the present time, a number of commercial houses have been licensed for the production and distribution of formalin-inactivated Newcastle disease virus vaccine. Promising results have been obtained in field trials with this product, yet there is much room for improvement because of the short-lived immunity which it produces. In general, immunization against Newcastle disease with either of the previously mentioned vaccines is more or less in the experimental stage in the United States.

LITERATURE

One method for vaccine production is that of modifying the virus by serial passage through the egg embryo. Iyer and Dobson,[1] with a limited number of birds, found that egg-passaged virus (forty-first passage) successfully immunized fowls. One of the birds was killed five days later, and material from the spleen was inoculated into a susceptible bird. The bird not only showed no visible symptoms of the disease but was successfully immunized. Burnet and Ferry[2] indicated there was no modification of the virus after at least 12 passages. Coronel [3] reported that 100 or more serial passages of the virus in embryonic tissue did not attenuate sufficiently to make it a safe vaccine for adult birds. Komarov and Goldschmid[4, 5] reported on the reduction in virulence of Newcastle virus by intracerebral passage in ducklings. Other investigators[6, 7] have also reported on experiments with live virus vaccines for immunization against Newcastle disease.

The second method employed for Newcastle disease vaccine is the production of attentuated or inactivated vaccines. Brandly and associates[8] showed that an inactivated virus vaccine, properly administered, was of definite value in the control of Newcastle disease. Their work on the inherited immunity, of four to five weeks duration, of chicks from Newcastle disease immune dams is of paramount importance in an immunization program against the disease. The status of the laying flocks may easily be obtained by conducting neutralization or hemagglutination-inhibition tests on representative serum samples from hens in the flocks. Iyer[9] found that a satisfactory vaccine for Newcastle disease may be prepared from egg-propagated virus by treatment with crystal violet, but that formalin had no apparent value in the preparation of a suitable vaccine from egg virus. Acevedo and Mendoza[10] produced a satisfactory protective vaccine from chicken-embryo virus treated with crystal violet. Beach[11] reports that a vaccine prepared from infected chicken embryos and rendered non-infective with formalin did not entirely prevent Newcastle disease (avian pneumoencephalitis) but enabled the chickens to go through a virulent outbreak of the disease with relatively low mortality. In addition, the effect of the disease on egg production was lessened by the vaccination. Coronel[3] produced a highly potent vaccine by using alumina cream as an absorbent and emulsifier with formalin as an attenuating agent. Cooper[12] and Farinas[13] in experiments

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Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, United States Department of Agriculture, Washington, D. C. (Schoening, Osteen, Legenhausen, and Hall); Branch Pathological Laboratory, Denver, Colo. (Anderson).

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with chloroform, toluol, tricresol, and phenol reported the vaccines as valueless or disappointing. Haddow[14] reported that formalinized vaccines were helpful, but the reaction was too irregular to permit field trials. Traub[15, 16] has also reported on the effectiveness of formalinized vaccines in varying doses. Others[17, 18, 19, 20] have recorded their results with the use of inactivated virus vaccines.

The experiment reported in this paper was conducted to determine whether broilers vaccinated at the age of 3 or 4 weeks would be protected against Newcastle disease virus by formalin-inactivated, commercially prepared vaccines.

MATERIALS AND METHODS

Plant Selected for Test.—The experiment was carried out in a large broiler plant on the Del-Mar-Va peninsula, made up of a part of Delaware, Maryland, and Virginia, between the Chesapeake Bay and the Atlantic Ocean. The disease was known to exist in most of the broiler houses of this plant for at least two years previously, as determined by clinical symptoms, virus isolation, and serum-neutralization tests. The economic value of the experiment was determined by the net profit of the vaccinated houses as compared with that of the control house. The broiler houses of the plant selected were characteristic of this area. The feed room was near the center of each house. The individual pens, eight to ten in number, extended out as a wing from each side of the feed room. The pens averaged about 16 by 32 ft. and were separated by a board partition running from the floor to the ceiling. A swinging door, at the front of each of these partitions, enabled the caretaker with his feed carrier, moving on an overhead rail, to pass from one pen to the next to feed and water the chicks and tend the coal brooder stoves. The doors to the pens usually remained open, allowing the chicks to run from one pen to the next, especially when the feed carrier passed through. Each pen had two brooder stoves and started with 1,000 chicks. The three houses were well separated from other broiler houses, the nearest being about a quarter of a mile.

Houses B and C had ten pens on each side of the feed room. House A had eight pens on the west side and nine pens on the east side of the feed room. At the rate of 1,000 chicks to a pen, the houses were started with the following number of chicks: B and C, 20,000 each; A, 17,000.

Newcastle disease occurred in the chickens occupying the houses immediately preceding the present flocks. Diseases and the number of times diagnosed in the experimental flocks up to date of vaccination were as follows:

	Cholera	Typhoid	Pullorum Disease
House A	1	1	7
House B	0	0	0
House C	1	2	24

Most of the local outside flocks had a history of a drop in egg production and of off-colored, soft-shelled eggs, which are suggestive of Newcastle disease infection.

Chickens.—The chicks in house A were from eggs supplied by outside local farms. Most of the eggs were from Newcastle-immune donors; about 10 per cent were not. House B was stocked with chicks from eggs from the company's own laying flocks, some of which were located on the immediate premises and known to be Newcastle immune as a result of natural infection and recovery. The remaining laying flocks were located in another state and their Newcastle disease experience was unknown. The house C chicks were derived from the above three sources. All chicks were hatched in the company's local hatchery.

All the chicks in the experiment were placed in their respective houses when 1 day old. House A started with 17,000 chicks and had a total mortality of 346 (2.03%) from various causes up to the date of vaccination. The remaining birds were then 22 to 23 days old and were apparently normal. House B started with 20,000 chicks and had a total mortality of 1,222 (5.10%) up to the date of vaccination. The remaining birds in this house were now 28 to 29 days old. When vaccination was begun, the birds in the east side of the house were experiencing a rather severe respiratory disease of about two days' duration. Control house C, started with 20,000 chicks, had a total mortality of 1,228 (6.14%) up to the time the birds were vaccinated in houses A and B. The birds in this entire house were experiencing a severe respiratory disease of two or three days' duration at this time, the east side being extensively affected.

Serum from blood samples obtained from a representative number of birds from each of the three houses at the time of vaccination failed to show significant titers against Newcastle disease virus. Nor was this virus recovered from tissues obtained from sick birds in the east side of house B and from house C at the time vaccination was started.

Vaccines.—The vaccines used were formalin-inactivated, commercially produced products. Vaccine X consisted of a 25 per cent saline suspension of chicken embryo-propagated virus. Vaccine Y consisted of a 50 per cent saline suspension of chicken embryo-propagated virus. The cost of the vaccine per bird was 1.33 cents, and the labor cost of vaccination was 0.4 cent per bird. Total cost of vaccine and its administration was 1.73 cents per bird.

Vaccination Procedure.—The vaccinating crew consisted of women regularly employed by various broiler plants for the purpose of vaccinating birds for laryngotracheitis and fowl pox. To avoid introduction of disease into the experimental flock by the vaccinating crew, boots and surgical robes were worn by each person.

Vaccination was begun with automatic vaccinating equipment but was shortly discarded in favor of 10-cc. glass syringes. The automatic equipment proved too awkward to operate and also was easily gummed by the vaccine. A catching pen consisting of frame sections 2½ ft. high and 4 ft. long covered with chicken wire and connected by hinges (which made a total length of 16 ft. when unfolded) was used to corner the birds in each pen.

The crew was organized so that 2 women picked the birds up out of the catching pen and handed them singly to 1 of 4 women who held the bird with a leg outstretched while 1 cc. of vaccine was injected intramuscularly in the leg. As vaccination was completed, the bird was dropped to the floor. Two women performed the vaccination while another cleaned and filled the syringes. Thus, a vaccinating team was composed of 9 members. This arrangement permitted the vaccination of 1,200 to 1,600 birds per hour.

Other than a slight limp observed the following day in some of the birds, no untoward results were encountered.

EXPERIMENTAL RESULTS

House A.—The 16,654 birds remaining in house A were vaccinated when they were 22 and 23 days old. From all appearances, they were normal at the time they were vaccinated. However, five days following vaccination, evidence of a respiratory disturbance was noted, and within five days morbidity became exceedingly high, but mortality was very light. Within the following week, morbidity subsided and the birds appeared to improve. Respiratory symptoms were not entirely absent up to the time the flock was sold, but there was a gradual decline of these symptoms to a low percentage until the end of fourteen weeks, when the birds were moved to market.

The most serious losses in the house were caused by coccidiosis, as diagnosed by postmortem examination, which was present between the fifteenth and twenty-first days after vaccination and when the birds were 38 to 44 days old. Total losses from the disease for this period were approximately 450, with a peak loss of 180 for any one day. The birds were treated with sulfamethazine and the outbreak was stopped. No further trouble from coccidiosis occurred.

Manifestations of the nervous form of Newcastle disease, characterized by torticollis, walking in circles, or walking backwards, were first noticed at 8 weeks of age. At sixteen days postvaccination, representative serum samples from birds in this house did not show significant titers against Newcastle virus. Yet five days later, a titer of 10^5 was obtained from samples from this group, which certainly would indicate Newcastle virus infection in the birds ten or more days previously. Past experiences with serum-neutralization titers of chickens vaccinated with formalin-inactivated Newcastle vaccine have shown that such high titers were not obtained as a result of such vaccination. It, therefore, appears that the chickens in this house had undergone an attack of Newcastle disease virus infection after they were vaccinated. No difference was noted in the protective qualities of vaccines X and Y, used in the east and west sides of the house, respectively.

All the birds, except culls, were sold for 30.25 cents a pound and were moved in the week of July 7, 1947, when they were 14 weeks of age. The buyers stated that the birds in this house were of good quality. Following is a complete report of mortality, feed consumption, expenses, and income for house A:

Total mortality	1,800
Total culls	145
Number of birds sold	14,706
Pounds, net weight, sold	43,081
Per cent mortality	10.5
Average weight of chickens, pounds	2.93
Total corn and mash fed, tons	90.20
Gross income (including culls sold for 10 cents a lb.)	$13,054.30

Expenses

Feed	$ 9,081.50
Labor (caretaker)	414.30
Newcastle disease vaccination	288.10
Chicks (10 cents each)	1,700.00
Laryngotracheitis vaccination	200.00
Coal	289.00
Total expenses	$11,972.90
Net profit	1,081.40

House B.—The 18,778 birds remaining in house B were vaccinated on the same dates,

TABLE I—Results of Serum-Neutralization Tests in Embryonating Chicken Eggs for the Viruses of Newcastle Disease and Infectious Bronchitis

Date blood collected	Age of birds (weeks)	Time after vaccination (days)	Time after vaccination (weeks)	House from which serum was obtained	Embryo m.l.d. Newcastle virus neutralized	Embryo m.l.d. bronchitis virus neutralized
4- 8-47	4	5		A-E	N.	
	4	5		A-W	N.	
	5	5		B-E	N.	
	5	5		B-W	N.	
	5	5		C-E	N.	
	5	5		C-W	N.	
5- 8-47	6	15		A-E	N.	10^1
	6	15		A-W	N.	1
	7	15		B-E	10^3	1^{+3}
	7	15				
	7	15		C-E	10^3	1^{+1}
	7	15		C-W	10^4	10^3
5-21-47	8		4	A-E	10^5	
	8		4	A-W	10^6	
	8		4	B-E	10^5	
	8		4	B-W	10^5	
	8		4	C-E	10^5	
	8		4	C-W	10^5	
5-27-47	8		5	A-E	10^5	10^4
	8		5	A-W	10^5	10^4
	9		5	B-E	10^6	10^4
	9		5	B-W	10^5	10^3
	9		5	C-E	10^5	10^4
	9		5	C-W	10^5	10^4
6- 3-47	9		6	A-E	10^5	10^4
	9		6	A-W	10^5	10^3
	10		6	B-E	10^6	10^3
	10		6	B-W	10^6	10^3
	10		6	C-E	10^5	10^4
	10		6	C-W	10^5	10^4
6-10-47	10		7	A-E	10^5	10^4
	10		7	A-W	10^4	10^4
	11		7	B-E	10^5	10^4
	11		7	B-W	10^4	10^4
	11		7	C-E	10^5	10^4
	11		7	C-W	10^4	10^3
6-17-47	11		8	A-E	10^6	
	11		8	A-W	10^5	
	12		8	B-E	10^5	
	12		8	B-W	10^5	
	12		8	C-E	10^4	
	12		8	C-W	10^5	
6-24-47	12		9	A-E	10^5	
	12		9	A-W	10^5	
	13		9	B-E	10^4	
	13		9	B-W	10^5	
	13		9	C-E	10^6	
	13		9	C-W	10^5	

E = East; W = West side of the house; N indicates less than 10^2 m.l.d.'s of Newcastle virus neutralized.

Apr. 22 and 23, 1947, as those in house A, and were 28 and 29 days old. Birds in the east side of this house were experiencing an outbreak of Newcastle disease and/or infectious bronchitis at the time of vaccination. The percentage of respiratory symptoms was high at this time, and the mortality on the second day of vaccination was 95 per day for this side of the house as compared with an average of 2 or 3 per day for the west side. On the third day, the mortality on the east side mounted to 289 birds, but during the next six days, it dropped to that of the west side, 7 to 12 birds, and continued thereabouts until sale. Sixteen to eighteen days following vaccination, birds on the west side of the house appeared to be "breaking" as evidenced by a rapid increase in morbidity as well as a rise in mortality overnight to 22 birds. This was the total extent of the "break" on this side and mortality dropped suddenly to 5 birds within three days, although morbidity declined more slowly. Except for slight losses due to coccidiosis, substantiated by postmortem examination, mortality of this house remained normal for the rest of the period.

Nervous manifestations characteristic of Newcastle disease were first noticed two weeks after vaccination on the east side and

four weeks after vaccination on the west side. Composite serum samples taken six days after vaccination gave a negative response to the serum neutralization test for Newcastle disease. Yet, samples obtained sixteen, twenty-one, and twenty-six days after vaccination yielded positive titers of 10^5 or more, indicating previous Newcastle virus infection. Again, as in house A, there was no difference noted in the protective qualities of vaccines X and Y used in the east and west sides of this house, respectively.

The birds, except culls, were sold for 30 cents a pound in the week of June 30, 1947, when they were 14 weeks old. Following is the report of mortality, feed consumption, expenses, and income for house B:

Total mortality	3,426
Total culls	200
Number of birds sold	16,454
Pounds, net weight, sold	50,080
Per cent mortality	12.1
Average weight of chickens, pounds	3.04
Total corn and mash fed, tons	106.7
Gross income (including culls sold for 10 cents a lb.)	$15,065.60

Expenses

Feed	$ 9,996.80
Labor (caretaker)	520.00
Newcastle disease vaccination	328.63
Chicks (10 cents each)	2,000.00
Laryngotracheitis vaccination	200.00
Coal	340.00
Total expenses	$13,385.43
Net profit	1,680.17

House C (Controls).—Chicks were put in house C on March 25, 1947, and were not vaccinated. Birds in this house had a respiratory disturbance at the time the birds in the other houses were vaccinated. The outbreak of Newcastle disease and/or infectious bronchitis was more extensive on the east side of the house, reaching a peak daily loss of 683 birds at 36 days of age. The west side of the house also had a Newcastle and/or infectious bronchitis disease outbreak, but it never resulted in sudden, severe losses. The outbreak, of a chronic form, lasted over a longer period than in the vaccinated groups, the daily losses averaging about 21 for the month of May.

Nervous manifestations characteristic of Newcastle disease were first noticed the week of May 5, 1947, following the outbreak, when the birds were 7 weeks old, three weeks following vaccination of birds in houses A and B. Birds in house C were also affected with coccidiosis following the Newcastle and/or infectious bronchitis outbreak, and losses were comparatively high, mounting to 133 for one day. The birds improved during June up to the time they were sold in July for 30.5 cents a pound at approximately 16 weeks of age.

Composite serum samples from birds in this house did not show significant protective titers against Newcastle virus on the sixth day after vaccination. Serum protective titers sixteen days after vaccination, however, were significant of previous infection of the birds with Newcastle virus in that 10^4 and 10^5 titers were obtained.

Following is the mortality, feed consumption, expenses, and income for house C:

Total mortality	7,264
Total culls	415
Number of birds sold	12,714
Pounds, net weight, sold	39,129
Per cent mortality	36
Average weight of chickens, pounds	3.065
Total corn and mash fed, tons	96.25
Gross income (including culls sold for 10 cents a lb.)	$12,006.65

Expenses

Feed	$ 9,023.25
Labor (caretaker)	520.00
Chicks (10 cents each)	2,000.00
Laryngotracheitis vaccination	200.00
Coal	340.00
Total expenses	$12,083.25
Loss	76.60

DISCUSSION

Symptoms of the acute stage of disease in each of the three houses were suggestive of both infectious bronchitis and Newcastle disease. Usually within ten days to two weeks following acute respiratory symptoms, nervous symptoms associated with Newcastle virus infection were observed. Postmortem findings on a great number of birds examined revealed the following lesions: Some of the birds had congested lungs with cloudy air sacs and bronchi partially or completely occluded with a mucopurulent exudate. Many of the birds showed

cloudy air sacs with no congestion of the lungs. With progressing chronicity of the disease, a large number of the air sacs showed an accumulation of a cheesy exudate, whereas the number of cloudy air sacs alone decreased.

The above lesions might be attributable to either Newcastle or bronchitis virus infection. From the results of serum-neutralization tests against both the virus of Newcastle disease and infectious bronchitis, it is apparent that the two diseases were present concurrently in each of the three houses. Table 1 shows the results of these tests. As already stated, in house A the birds were apparently normal at vaccination time, whereas birds in the east side of house B and all the birds in house C had a rather severe respiratory disturbance at this time. Yet five days postvaccination, the birds in house A showed evidence of a severe respiratory infection. Based on previous experimental data, the assumption is made that Newcastle disease virus was producing the symptoms in houses B and C at the time of vaccination of the birds in house B, and in house A five days following vaccination. In houses B and C, significant protective titers suggestive of Newcastle virus infection were obtained from serum samples from birds in these houses at sixteen days postvaccination, while in house A significant titers did not show until the twenty-first day postvaccination. As previously stated, birds vaccinated with formalin-inactivated vaccines rarely, if ever, produced significant neutralizing titers against the specific virus. The table shows that, at the same time, significant neutralizing titers against infectious bronchitis virus were obtained from birds in either one side or the other of the houses in the experimental group, thus suggesting the probability that the two viruses were operating concurrently.

In each of the houses, a discrepancy exists between the number of chicks placed in a given house and the number sold, plus total mortality. This is due to occasional losses from rats, cats, dogs, and wild animals such as foxes and skunks. These differences, obviously, cannot be accurately tabulated, although it is felt they were not sufficient to affect the experiment seriously. Rather heavy losses were sustained from coccidiosis in houses A and C, whereas slight losses from this disease were experienced in house B. Losses from diseases other than Newcastle and other causes generally in the plant where this experiment was conducted are usually considered to be about 10 per cent. If this is correct, the losses from Newcastle disease for house A would be 0.5 per cent, with an over-all loss of 10.5 per cent. The losses for house B from Newcastle disease would be 2.10 per cent, with an over-all loss of 12.1 per cent. The losses in the unvaccinated birds in house C from Newcastle disease would be 26 per cent, with an over-all loss of 36 per cent. Birds in houses A and B, which were vaccinated against Newcastle disease, yielded a financial profit, even though bronchitis infection was present, whereas birds in the unvaccinated group in house C were a financial loss.

SUMMARY AND CONCLUSIONS

Results are reported on the vaccination of 37,000 chicks in a broiler plant against Newcastle disease with commercially produced killed virus vaccine, using an additional 20,000 chicks as unvaccinated controls.

Vaccination did not entirely prevent the disease but enabled vaccinated birds to withstand a severe infection with relatively small losses as compared with the unvaccinated controls.

The final results show that, in this instance, chicks vaccinated against Newcastle disease with a formalin-inactivated vaccine yielded the owner a profit, whereas the unvaccinated controls were a financial loss.

Vaccination with this type of vaccine is a practical procedure.

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Salmonella Infections in Mink and Ferrets

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IN THE PAST few years, laboratory workers making diagnoses for the fur animal industry have suspected members of the genus Salmonella as one of the etiologic agents of what the fur farmer terms food poisoning. This is a rather vague term used by the industry to describe a multiplicity of conditions, one of which is gastroenteritis.

The reports of Salmonella occurring in cases of gastroenteritis, with respect to the Carnivora in general, have been vague and infrequent. Early work, before Salmonella typing was established, is somewhat open to question, because of the great similarity between members of the genus Proteus and the genus Salmonella. Some recent reports appearing in lay journals have not been properly substantiated.

With these facts in mind, the authors thought it desirable to test the primary etiologic relationship of Salmonella to the gastroenteritis syndrome. In an effort to simulate ranch conditions, *per os* infection was considered desirable. Preliminary trials showed mink and ferrets to be very refractory to oral exposure. Therefore, trials were planned to lower the resistance of the animals by various methods which might be duplicated under ranch conditions. Trials were also made by feeding naturally and experimentally infected carcasses as a means of exposure. Some animals were inoculated parenterally to provide material for pathologic comparison.

LITERATURE

Reported Isolations from Mink and Ferrets.—In a review of the literature, it appears that positive isolations from mink have been infrequent. According to the literature, no isolations have been reported from ferrets. One of the first isolations of Salmonella from mink was made by Sprehn,[1] who isolated the type, Breslaviense.* Meyn[2] reported finding the same organism from cases of paratyphoid in mink. Gray[3] observed that infectious gastroenteritis was often found in mink and that the etiologic agent was *Salmonella aertrycke* of the Gärtner group of *Bacterium breslau*. Meyer, Gmeiner, and Knorpp, cited by Hutyra, Marek, and Manninger,[4] reported considerable losses on mink farms due to paratyphoid infection. The species and variety names were not given. Benedict *et al.*[5] isolated *Salmonella pullorum* in cases of distemper. Hoyt[6] recovered *Salmonella choleraesuis* var. *kunzendorf* and *Salmonella typhimurium* from cases of catarrhal enteritis. Momberg-Jørgensen[7] in Denmark reported that Salmonella infections of mink were rare and sporadic and that the causal agent was *S. typhimurium*. Chaddock[8] found *Salmonella suipestifer* in an outbreak of paratyphoid. Phillips[9] recovered *Salmonella montevideo* in an outbreak of hemorrhagic enteritis. He reported a mortality of approximately 50 out of a total of 350 mink.

Hartsough[10] described an unusual outbreak, involving pregnant females, which occurred in the spring of 1946 and of 1947. There were few deaths, but the predominant symptom was abortion. A number of aborting females were sacrificed and examined bacteriologically, and in every instance *S. choleraesuis* var. *kunzendorf* was isolated from the uterus and unexpelled fetuses. He believed that the source of infection in both of these outbreaks was infected pork livers.

Recently, Kennedy[11] reviewed his experiences with Salmonella infections in mink. A personal communication from Kennedy[12] stated that in most cases *S. typhimurium* was isolated. He has also isolated *S. choleraesuis* var. *kunzendorf* and *Salmonella enteritidis*. A check list supplied by Edwards[13] showed that *Salmonella newport*, *S. choleraesuis* var. *kunzendorf*, *Salmonella bareilly*, and *S. pullorum* had been isolated in cases of gastroenteritis in mink.

Experimental Infections.—Because experimental infection of mink has been infrequent, it was of interest to learn what attempts had been made to infect foxes, dogs, and cats.

Some of the earlier European work has been reviewed by Momberg-Jørgensen,[14] who cited several experiments. Riëdmuller and Saxer, in 1930, experimentally infected a Silver fox by feeding the viscera and meat of a rabbit that had been artificially infected with *S. suipestifer*. After an incubation period of seven days, the fox became ill and died shortly thereafter from paratyphoid. Schoop, in 1931, gave large

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In this investigation, the authors made all of their measurements in ml.; 1 ml. is equal to 1.000027 cc.

*Nomenclature of original sources is used in this manuscript to avoid possible errors in conversion to new nomenclature based on serology.

doses of Gärtner cultures to 7 young Red foxes for one week with no detrimental effect. Again in 1931, Schoop fed cultures and spontaneously infected calf viscera to 3 older foxes with no success. Dedié fed a fox for two days with beef which was artificially infected by injection with the Gärtner bacillus without producing any symptoms in the fox. Olsson concluded that it is very difficult to produce the disease experimentally in foxes by the feeding of bacterial cultures or spontaneously infected organs.

Collins[15] described an outbreak of distemper in foxes in which a member of the Salmonella genus was isolated. Foxes and mink were fed quantities of a broth culture made from this strain. No detrimental effect was observed on the animals when they were fed the culture in the diet or when the diets were incubated with the culture and subsequently fed. On the basis of this experiment, Collins felt that in order for the Salmonella to show its enteritic effect, it must be preceded or accompanied by the virus of distemper.

Helgebostad[16] cited the work of Brant and Hellesnes. These men added a virulent culture of Salmonella, which had caused a paratyphoid epizoötic among foxes, to the food of healthy foxes. Another group of foxes was given the virus of distemper and fed food which similarly had virulent Salmonella added to it. The nondistemper group showed no symptoms of an infection. The other group which received both the virus and cultures succumbed.

Van Ermengem[17] described a *S. enteritidis* infection in a man which proved fatal. A mixture of liver and kidney was taken from this man at necropsy and injected subcutaneously, intraperitoneally, and by stomach tube into guinea pigs, rabbits, mice, rats, and dogs. All died in a short time except the 2 dogs.

Rettger, Slanetz, and McAlpine[18] gave cultures of *S. pullorum* along with egg yolks, infected with the same organisms, to young rabbits and kittens. The kittens exhibited a diarrhea approximately three days after the experimental feeding, which persisted for three days longer. At the end of the feeding trials, the kittens were sacrificed. A histopathologic examination was not made and no gross lesions were observed. Agglutination tests were made on the serums of the kittens, but no reactions were noted. They did not isolate *S. pullorum* from the blood or internal organs, and they concluded from this finding that the intestinal disturbance was caused by bacterial toxins. While there were no fatalities among the kittens, 18 of the 21 rabbits died. *Salmonella pullorum* was isolated in every instance from the blood of those rabbits which died.

Sherwood[19] found that cats possess almost solid immunity to strains of Salmonella given *per os*. He did not give the species and variety names; however, a personal communication from Sherwood[20] revealed that *S. enteritidis* was the test pathogen.

Coburn[21] inoculated ferrets and mink with a strain of *S. typhimurium*, which was found pathogenic for both of these carnivores.

Part I

MATERIALS AND METHODS

Bacteria.—In this investigation, three members of the genus Salmonella were used for experimental purposes. The culture of *S. enteritidis* (IX, XII: g.m.:-) was obtained through the courtesy of Dr. Charles Drake of the Department of Bacteriology, State College of Washington. This particular strain was recently isolated from a rat which had been killed and was used in the preliminary trials, because rats frequently are found on mink farms. *Salmonella newport* (VI, VIII... e,h-1,2,3. . .) and *S. choleraesuis* var. *kunzendorf* (VI, VII...: c-1,5...) were obtained from Dr. P. R. Edwards of the Department of Animal Pathology, University of Kentucky. These species were used because they were originally isolated from mink showing a gastroenteritis.

Virus.—The strain of distemper virus was obtained from a young dog exhibiting clinical neurotropic distemper. The history was typical. The dog showed catarrhal symptoms with an accompanying high temperature, which was followed by nervous symptoms ten days after the initial attack. The dog was sacrificed and the spleen was removed aseptically, weighed, and immediately placed in a sterile Waring blendor, which was chilled before using. A 10 per cent suspension in sterile, chilled saline was prepared. The blendor was run at half-minute intervals to prevent excessive heat formation by the blades. After the splenic trabeculae were broken down sufficiently, the suspension was placed in a sterile, chilled bottle and inoculated immediately into the experimental animals. A loopful of the suspension streaked on SS agar plates and inoculated into tryptose phosphate broth (Difco) failed to reveal any bacterial growth.

Isolation and Preparation of Cultures.—The isolation of Salmonella from infected animals and those animals acting as carriers was very difficult because of the large numbers of Proteus spp. present in the normal mink and ferret intestines. Various differential mediums and enrichment broths were used, including selenite-F, tetrathionate broth, Ruy's fluid, SS agar, and MacConkey agar. Ruy's fluid inhibited the growth of Proteus spp. to some extent and was used for that purpose. Selenite-F and tetrathionate broth were useless in this regard. SS agar was the medium of choice for the isolation of Salmonella; it, however, will

also support an excellent growth of Proteus spp. MacConkey agar was not as effective in preliminary trials and consequently was not used. Urease test medium (BBL) was very helpful in the differentiation of Salmonella from Proteus.

To identify species of Salmonella from the feces or gastrointestinal tract of the experimental animals, the following procedure was used. On the first day, an SS agar plate was streaked directly from the feces or intestinal contents and 1 Gm. of feces inoculated into 10 cc. of tetrathionate broth and 10 cc. of Ruy's fluid. On the second day, the original SS agar plate was examined, and two additional SS agar plates streaked from the two enrichment broths. Clear colonies appearing on the plates were inoculated into urease medium and Kligler's iron agar for further differentiation.

Tryptose phosphate broth was selected as the medium for the preparation of all cultures to be fed or used for inoculating purposes. It was believed that this medium would best supply the nutritive requirements and maintain the virulence of the bacteria used.

When the organisms were to be used for infection, the following method for increasing the virulence was used. Smooth colonies were selected from the streaked plates and inoculated into tryptose phosphate broth, which was incubated for twenty-four hours at 37.5 C. A volume of 0.1 cc. of this bacterial suspension was inoculated intraperitoneally into a Swiss mouse. Invariably, the mouse would die within twenty-four hours. The peritoneal cavity was opened aseptically and a loopful of the resulting exudate removed. Inoculation was then made into tryptose phosphate broth, incubated, and carried through another mouse and a guinea pig in the same manner. During this procedure, all cultures were constantly checked for purity. For some unknown reason, the virulence of *S. choleraesuis* var. *kunzendorf* was reduced after the first trials and could not be raised sufficiently to warrant using it in the later experiments. *Salmonella newport* maintained its virulence for mice and guinea pigs and was used as the pathogen for the greater part of the experimental work. The cultures used for the experimental feeding were incubated for twenty-four hours at 37.5 C. After incubation, they were immediately taken to the Bureau of Animal Industry Experimental Animal Unit, and mixed with the diet in a large enamel tray. The diet was prewarmed to a temperature of about 37 C. in order to prevent the possibility of the low temperature of the food destroying part, or reducing the virulence, of the inoculum. The regular diet was stored at a temperature approximating 4 C.

Histopathology.—Tissues removed from the experimental animals were placed in Bouin's fluid for fixing. The sections were stained with hematoxylin and eosin. For the demonstration of inclusion bodies in the bladder and trachea smears, the hematoxylin-S3 stain was employed.[23]

Animals.—Stock mink and ferrets from the Bureau of Animal Industry herd at the Fur Animal Disease Research Laboratory, Pullman, Wash., were used for the experimental work. When the animals were selected, their feces were cultured by the method described above, and none of the animals were found to be carriers of any Salmonella organisms. There had been no history of any Salmonella infections in the animal unit. This was the first time that Salmonella organisms had been introduced into any of the experimental animals. It was believed, therefore, that the animals had had no opportunity to develop any immunity to these organisms. The animals were housed in furring pens in batteries of 3 or 4. The pens had wire bottoms and contained no nest boxes. The regular diet consisted of horse meat (85%) and commercial cereal (15%), supplemented with a commercial fish oil. Each animal was fed approximately 7½ oz. of this combination per day; water was available at all times. All the animals were adults except those in experiment 1. Both males and females of the respective species were used.

The experiments were conducted from the middle of August through January. It was found that cold weather was not desirable, both from the standpoint of feeding and the effect on the animals themselves. The food froze because the animals would not eat promptly after feeding. Besides causing irregular food intake, this may have led to partial destruction of the contained inoculum. Instances where this occurred will be discussed under the specific experimental animal. Most animals, however, ate the food-inoculum combination readily.

EXPERIMENTAL

Experiment 1.—In this preliminary trial, a culture of *S. enteritidis* was used to determine its pathogenicity for young mink and ferrets. These animals were approximately 3 to 4 months old. Infection of the animals was attempted by feeding inoculum in the diet and by intraperitoneal injections.

Two mink and 2 ferrets were given 0.25 cc. of the test pathogen in broth intraperitoneally. No prodromal symptoms were noted for the remainder of the day, and the next morning all the animals were dead. Death had occurred within twenty hours. Postmortem examination showed a diffuse, dull, reddened peritoneum, injection of the serosal vessels, and a peritoneal exudate that was thin and serous. The liver, kidney, and heart appeared slightly enlarged and pale. The rest of the organs appeared normal grossly. All the animals pre-

sented essentially the same macroscopic lesions. No histopathologic material was collected. *Salmonella enteritidis* was isolated from the peritoneal exudate in each instance.

Smears made from the bladder and trachea were negative for the inclusion bodies of distemper.

An attempt to infect the animals orally was then tried. Two mink and 2 ferrets were used in this group. For seven days, they were given 50 cc. of the test pathogen in broth daily mixed with their normal diet. The animals ate the food-inoculum combination readily, and at the end of the trial they appeared to be thriving. Their feces were checked each day during the experimental feeding and three days subsequent to the last feeding. Each time, a few clear colonies were found on the SS agar plates, which were urease-negative. Identification was not carried further, as it was presumed that these colonies were Salmonella.

Experiment 2.—Since no infection resulted in the preceding trial, it was thought the lowering of the resistance of the host would allow the organisms to invade the tissues.

Some fur breeders, through ignorance or for financial reasons, maintain their animals in a low state of nutrition. Salmonellosis is frequently reported on these ranches. In order to simulate this condition, 9 mink (A-1 through A-9) and 9 ferrets (AF-1 through AF-9) were placed on half-rations for an extended period. They received approximately 3¾ oz. per day, and were not fed every seventh day. After a month had passed, the animals were emaciated, nervous, chewed their tails, and had dull, ragged-looking guard hairs. Probably most of them would have died had there been a sudden change of temperature with the weather becoming colder.

On the twenty-sixth day, 3 mink (A-1, A-2, and A-3) and 3 ferrets (AF-1, AF-2, and AF-3) were fed 100 cc. of *S. newport* culture in their feed. One mink (A-2) and 1 ferret (AF-3) became infected.

On the first day after feeding, the infected ferret showed lassitude, anorexia, and muscular trembling, and on the second day, it was passing blood in the feces. On the third day, the animal was moribund and was found dead at 8 a.m. on the following morning. The infected mink displayed symptoms very similar to those of the ferret just described. The only difference noted was that the mink preferred to lie tightly curled in its water dish, which is indicative of a rise in temperature. No symptoms were observed in control mink A-7, A-8, and A-9, or control ferrets AF-7, AF-8, and AF-9.

The macroscopic examinations were made within fifteen hours after death. Both of the animals were extremely emaciated and had ragged coats. Mink A-2 displayed an agonal intussusception of the colon with prolapse. Examination of the peritoneal cavity showed no perirenal adipose tissue. A few petechial hemorrhages were present on the parietal peritoneum, and the serosal vessels were injected. The mesenteric nodes were not prominent. The spleen was not enlarged or mottled, and on section, was normal in color and consistency. The liver was smooth, light brown, and of average firmness. The gall bladder was distended. The gastric mucosa was covered with a thin layer of mucus, and the small intestine and anterior colon contained a considerable amount of reddish black material. The lungs and pleural cavity were normal in appearance. The kidneys were light in color, and the capsules stripped with ease. On section, the cortices and medullae presented no gross abnormalities. The urinary bladder was distended; no calculi were observed. The heart was dilated.

Microscopically, the spleen showed atrophy of the splenic follicles, which appeared small and sharply demarcated from the surrounding red pulp. The arteries of the splenic follicles were prominent and appeared hyperplastic. Their walls were markedly thickened with the increase arising chiefly in the mediums. No macrophagic or lymphocytic increase was noted in the red pulp.

The liver showed hyperemia of the central veins and branches of the portal veins. Small accumulations of macrophages were scattered in the parenchyma, not confined to any specific histologic locations. No coagulation necrosis was noted in the individual hepatic cells surrounding these accumulations.

The kidneys exhibited small focal macrophagic accumulations in the cortices. In some areas, the tubular epithelium in the medulla had an eosinophilic hue with nuclear pyknosis, evidence of beginning coagulation necrosis.

The pathologic changes were most marked in the gastrointestinal tract. In sections of the stomach, the mucosa had an overlying layer of blue-staining material which contained limited numbers of macrophages and polymorphonuclears. In some areas, the surface epithelium stained lighter, and extensive desquamation of the epithelium was noted. A limited infiltration of macrophages and lymphocytes with an occasional polymorphonuclear was observed near the surface in the connective tissue among the gastric pits. The serosal vessels were dilated and filled with the blood including many monocytes.

The changes in the small intestine and colon were more evident. The lumen was filled with red blood corpuscles in some sections, while in others, eosinophilic shreds and granular material overlaid the mucosa. Mucoid material was observed overlying the villi of the jejunum. In one region, the mucous membrane was

eroded down to the muscularis mucosa. No inflammatory response was noted around this erosion. There was a marked leucocytic infiltration of the mucosa of the small intestine and anterior colon. The infiltrating cells consisted of macrophages, lymphocytes, and a few polymorphonuclears. The submucosal vessels of the small intestine and colon were hyperemic.

In sections of the heart, some areas of cardiac muscle showed coagulation necrosis. The fibers had an eosinophilic hue, and the nuclei were pyknotic. The lungs displayed no microscopic abnormalities.

Salmonella newport was isolated in pure culture from the heart blood, spleen, liver, and bone marrow. It was also recovered from the small intestine and colon.

Impression smears made from the bladder and trachea were negative for the inclusion bodies of distemper.

The macroscopic examination of ferret AF-3 showed lesions very similar to those found in mink A-2. The peritoneal cavity displayed no excessive fluid, and the parietal peritoneum presented a normal appearance. The serosal vessels were hyperemic. The mesenteric nodes were not prominent. The spleen was not enlarged or discolored, and on section, was normal in consistency and color. The liver was normal in appearance, and of average firmness on section. The gall bladder was distended, and there were areas of biliary imbibition in the substance of the liver. The kidneys were light in color, and the capsules stripped with ease. The urinary bladder was distended. No changes were observed in the heart and lungs.

Macroscopic changes of particular interest were noted in the gastrointestinal tract. The stomach contained much mucus, intermixed with dark red, stringy material which adhered to the mucosa. The small intestine was filled with a large amount of dark red, semisolid material. A small amount of mucus was noted in the ileum. After the exudate was washed away, the mucosa of the stomach and small intestine was seen to be slightly reddened. The colon presented no mucosal abnormalities, and its lumen contained a few strands of dark red, stringy material.

Microscopically, the spleen showed no changes. No macrophagic or lymphocytic increase was observed in the red pulp. In sections of the kidney, the glomeruli were more cellular; the sections of the kidney were cut thicker than usual, and this fact may have accounted for the cellularity. Small focal accumulations of macrophages were observed in the cortices. An occasional pyknotic nucleus was noted in the tubular epithelium.

In the liver, the sinusoids were filled with blood to a greater degree than normally observed. The branches of the portal and central veins were also distended with blood. As in the kidney, small focal accumulations of macrophages were scattered in the parenchyma.

The most marked changes were found in the gastrointestinal tract. There was a large amount of mucus intermixed with large numbers of solidly packed red blood cells overlying the mucosa of the stomach. This exudate also contained a few mononuclears and bits of desquamated epithelium. A limited infiltration of macrophages and lymphocytes was noted near the surface in the connective tissue among the pits. The exudate in the small intestine consisted of mucoid material and red blood cells. In some areas, the tips of the villi had been desquamated and were lying free in the lumen. The villi of the ileum were edematous; in the connective tissue cores, pink granular material and fine threadlike strands were noted. Throughout the small intestine, there was a marked diffuse infiltration of the mucosa with macrophages and lymphocytes.

Salmonella newport was recovered in pure culture from the heart blood, spleen, liver, and bone marrow. It was also isolated from the small intestine and colon.

No inclusion bodies were noted in the bladder and tracheal epithelium.

On the thirtieth day of the depletion period, 3 mink (A-4, A-5, and A-6) and 3 ferrets (AF-4, AF-5, and AF-6) were each fed 100 cc. of *S. choleraesuis* var. *kunzendorf* in their feed. They experienced no apparent discomfort from this feeding, and the controls remained normal. For twenty-four days following this feeding, they remained on the reduced ration. On the twenty-fifth day, they were each fed 100 cc. of *S. choleraesuis* var. *kunzendorf* and the same amount on the following day. All feed was withheld for twenty-four hours, and they were each fed 100 cc. on the twenty-eighth and twenty-ninth days. Neither the effect of the short fast nor the large amount of inoculum (each animal received a total of 500 cc.) had any effect on the animals. The depleted controls remained normal.

The survivors of those which had been subjected to the feeding of *S. newport* (A-1, A-3, AF-1, and AF-2) were each given further culture feedings of 100 cc. on the thirty-fifth, thirty-seventh, and thirty-ninth days. Including their first feeding on the twenty-sixth day, each animal received a total of 400 cc. No symptoms were observed in these animals or the depleted controls A-7, A-8, A-9, AF-7, AF-8, and AF-9.

Newsom and Cross[23] found that by fasting lambs for twenty-four to seventy-two hours, they could cause death in these lambs by feeding Salmonella cultures. Consequently, since fasting of twenty-four hours with the subsequent feeding of cultures caused no infection in ferrets and mink, it was thought desirable

to fast a group of mink and ferrets for a longer period.

Mink A-1 and A-3 and ferrets AF-1 and AF-2 were subjected to a seventy-two-hour fast and fed 100 cc. of *S. newport* culture. No symptoms were noted, but the depleted control AF-8 succumbed. Postmortem examination of this animal showed extreme emaciation. Salmonella organisms were not isolated from the spleen and colon, and no distemper inclusions were demonstrated in the trachea or bladder smears.

Each time the cultures were administered to depleted animals, healthy stock mink and ferrets, which were fed the regular diet, were similarly fed the same cultures in identical amounts. Mink AM-50 and AM-51 and ferrets A-50 and A-51 were used as controls in the *S. newport* feedings; mink AM-52 and AM-53 and ferrets A-53 and A-54 were used as normal controls in the *S. choleraesuis* var. *kunzendorf* feedings. No symptoms were observed in this group during the entire study.

The feces of the depleted and control groups which had been fed Salmonella cultures were cultured after each experimental feeding and were found to contain organisms which were urease-negative. These were presumed to be Salmonella; detailed identification was not feasible.

Experiment 3.—Salmonellosis is thought to occur on ranches in which distemper has appeared and lowered the resistance of the survivors enabling the Salmonella organisms to establish an infection. In order to duplicate this situation, 9 mink (B-1 through B-9) and 9 ferrets (BF-1 through BF-9) were each inoculated in 2-cc. amounts with a splenic suspension made from a dog affected with distemper.

On the fifteenth day following the inoculations, 3 ferrets (BF-2, BF-6, and BF-8) displayed a slight squinting of the eyes with a serous exudate on the conjunctiva, followed by a loss of appetite, gluing of the upper and lower lids with a purulent exudate, and the formation of vesicles on the lips and nose. These vesicles soon ruptured, and were replaced by brown, flaky material. Although swelling of the feet was not marked, the syndrome was typical of distemper. These animals were sacrificed, as they could not be induced to eat, thus eliminating them for experimental feeding. Control animals with distemper were desired, and they were used for that purpose. No Salmonella organisms were isolated from these animals. Inclusion bodies were found in the bladder and tracheal epithelium.

On the twenty-sixth day after the inoculations, the mink had not shown an observable symptom of distemper. An attempt was made to utilize these animals (B-1 through B-9) by reinoculating them with 2 cc. of a 10 per cent liver-spleen suspension made from ferret BF-2. No symptoms of distemper were noted two months after the second inoculation; the animals were considered immune at that time and discarded. Assuming that a single strain of distemper virus was involved, it is possible that the first inoculation was virulent enough to cause the disease in ferrets, and acted as a vaccine for the mink, thus protecting them against future exposures.

Ferrets BF-1, BF-3, BF-4, BF-5, and BF-7 showed symptoms of distemper on the eighteenth to twenty-second days after the virus inoculations. They were then each subjected to cultures of *S. newport* in their diet. The cultures were given on successive days to eliminate the possibility of the animals forming any partial resistance. Ferret BF-9 did not show any clinical distemper and was discarded.

Ferrets BF-3 and BF-7 ate the culture-diet combination promptly; ferret BF-3 became accustomed to the feedings and preferred inoculated food to the regular ration. They were fed each day until they became moribund from the effect of the virus. Each animal received a total of 1,150 cc. Ferret BF-5 was fed in the same manner, and received a total of 650 cc.

During this trial, the weather became very cold and the food froze in a short time. Ferrets BF-1 and BF-4 did not eat rapidly enough; consequently, they received a total of only 250 cc. of culture. For five successive days the weather remained warm enough and the food did not freeze; on those days they received 50 cc. of culture per day.

None of the animals receiving the cultures showed any effects from the experimental feeding. They exhibited no diarrhea and died from the action of the virus.

Pathologic examination, both gross and microscopic, performed on ferrets BF-1, BF-3, BF-4, BF-5, and BF-7 showed lesions of distemper, none of which would suggest a secondary Salmonella infection.

The feces of these animals were plated during the trial. Clear colonies picked from SS agar plates were found to be urease-negative and were presumed to be Salmonella.

Experiment 4.—For purposes of pathologic comparison, *S. newport* was injected in 2-cc. amounts into the gracilis muscle of 2 adult mink and 3 adult ferrets. On the following day, the mink appeared restless, did not eat readily, and dragged the injected leg. The ferrets exhibited no symptoms other than the favoring of the inoculated leg. On the third day after the injections, the mink had no interest in their food and preferred to lie unmolested and tightly curled in the corner of the pen. The ferrets continued to eat well and showed no depression. The symptoms shown

by 1 mink continued to be more severe. This animal showed complete anorexia, frothing at the mouth, coma, and finally death on the ninth day after inoculation. The other mink displayed many of the same symptoms, without the extreme depression, and made a slow recovery. One of the ferrets on the seventh day after inoculation showed partial anorexia and slight depression, followed by symptoms not unlike those shown by the mink, and died on the eleventh day after injection. The remaining ferrets were unaffected.

Autopsies were performed within fourteen hours after death. The gross appearances of the dead mink and ferret were similar. When the peritoneal cavity was opened, the serous surfaces of the stomach and small intestine were reddened. There were no evidences of fibrinous exudation and no excessive fluid. The mesenteric lymph nodes were not prominent. The spleen was greatly enlarged, bluish red, and on section, was firm in consistency and presented a uniformly dark red appearance. The liver was increased in size, firm, and dark red; the cut surface displayed a mottled appearance of dark and light areas. The kidneys were apparently normal. The stomach, small intestine, and colon showed no mucosal changes other than a slight reddening. The lungs, heart, and pleural cavities presented a normal appearance. Histopathologic examination was made on the mink only.

In the mink, the microscopic feature of particular interest was observed in the spleen. The capsule was distended, and the trabeculae were not prominent. Many megakaryocytes, some degenerating, were scattered throughout the red pulp. The red pulp areas were dilated and contained principally macrophages arising through hyperplasia or accumulation from the peripheral blood stream. The splenic follicles were depleted, and the areas that had undergone depletion were lighter and the cells which were present looked very similar to young fibroblasts interlaced in a delicate network of fibers; necrosis was not evident in this area.

The liver cells showed considerable vacuolation, particularly at the periphery of the lobules, but not confined to this area. The sinusoids were distended with blood, the Kupffer cells prominent, and small focal areas of mononuclears with an occasional polymorphonuclear cell were observed at the hepatic trinities and scattered in the parenchyma. No areas of necrosis were seen.

The microscopic changes in the gastrointestinal tract were confined to the small intestine. The serosal vessels were dilated. A moderately diffuse infiltration of macrophages and lymphocytes with occasional polymorphonuclears was noted in the mucosa. The heart, kidneys, and lungs exhibited no outstanding changes.

Salmonella newport was isolated from the spleen and small intestine. Smears made from the bladder and trachea were negative for distemper inclusion bodies.

Part 2

MATERIALS AND METHODS

Bacteria.—*Salmonella newport* (VI, VIII...: e,h-1,2,3...) was selected as the test pathogen. This species was the same as used in part 1.

Near the termination of the experimental work, the veterinary school at Washington State College had an outbreak of salmonellosis in the mouse and guinea-pig colony. The etiologic agent was found to be *S. typhimurium* (IV, V, XII...: i-1,2,3...). The isolation was confirmed serologically by Dr. P. R. Edwards. In order to obtain further information as to whether material from a spontaneous outbreak would be infective, livers from 2 of the guinea pigs were fed to 2 mink.

Isolations and Preparation of Cultures.—In Part I, we found mediums commonly used for the isolation of the Salmonella group unsatisfactory as they permitted a luxuriant growth of Proteus organisms. The brilliant green agar of Kauffmann,[21] as modified by Morgenstern,[22] used with a preliminary enrichment in Kauffmann's modification of tetrathionate broth,[23] proved to be adequate in inhibiting the growth of Proteus spp. This method has also been used by Broh-Kahn[28] with good results.

Identification of Salmonella from the feces was carried out in the following manner. On the first day, 1 Gm. of feces was placed in 10 cc. of Kauffmann's tetrathionate broth. On the second day, a plate of Kauffmann's brilliant green agar was streaked from the broth. On the following day, typical colonies, which were pinkish white and opaque, were inoculated into Kligler tubes for further differentiation. If the suspected organism did not ferment lactose, it was placed in urease medium (BBL) to differentiate Proteus spp. and streaked on a 10 per cent lactose agar slant to differentiate from the paracolons. Colonies negative to the preceding were typed with a serum specific for *S. newport*. If agglutination occurred, the organisms were considered to be *S. newport*. For additional confirmation, colonies of this type were sent to Dr. P. R. Edwards.

Mice and guinea pigs were used to increase the virulence of the test organisms. When the culture killed young guinea pigs in less than eighteen hours, it was considered virulent. The culture was then transferred to a flask containing 1,000 cc. of tryptose phosphate broth and incubated for eighteen hours. The broth was then transferred to sterile tubes and centrifuged at 2,000 r.p.m. for thirty minutes. The supernatant fluid was removed, and the organisms were washed in sterile physiologic saline. After washing, the organisms were

suspended in 200 cc. of physiologic saline. This was used as the sheep inoculum.

Animals.—Since it is generally known that younger animals are more susceptible to enteric infections than older animals, the mink used in these trials were approximately 10 weeks old. None of the mink had had any previous contact with animals that had been given Salmonella cultures. Fecal cultures taken before the trials revealed no Salmonella. They were fed and housed under conditions similar to those mentioned in part 1 of this paper. Both males and females were used.

The sheep used was an old Columbia ewe and was obtained through the courtesy of Dr. R. W. Dougherty, of the Division of Veterinary Science. Fecal and blood cultures taken before the test inoculation were negative for Salmonella and other pathogens. The ewe had a normal temperature and no diarrhea.

EXPERIMENTAL

Experiment 5.—In this trial, an attempt was made to infect a group of 24 healthy young mink by feeding Salmonella-infected sheep muscle and viscera.

The sheep was given intravenously 125 cc. of the virulent *S. newport* suspension. Approximately three hours after administration, the sheep was frothing at the mouth and appeared depressed and dyspneic. Five hours after inoculation, it was moribund and showed epistaxis and a copious diarrhea. Death occurred approximately ten hours after inoculation.

TABLE 1—Results from Fecal Cultures Taken from Exposed Mink in Experiment 5

Bacteria	Number of colonies picked			
	Aug. 6	Aug. 9	Aug. 12	Aug. 16
Salmonella newport	43	42	41	33
Proteus spp.	4	3	1	7
Coliform spp.	0	0	5	5
Paracolon spp.	1	3	1	2
Total	48	48	48	47

Postmortem examination showed no outstanding changes. A few petechial hemorrhages were seen in the subcutaneous tissue and on the serous membranes. The mesenteric lymph glands were hemorrhagic. *Salmonella newport* was isolated in pure culture from the liver, spleen, heart blood, and skeletal muscle. Mice and guinea pigs were rapidly killed by intraperitoneal injection of a suspension of the growth.

With the exception of the hide, skeleton, and gastrointestinal tract, the entire sheep was ground through a meat chopper. To reduce the possibility of introducing excessive contamination, the stomach and the intestine were not used. After grinding, the meat that was not fed immediately was stored in large cans at a temperature of 5 C.

Meanwhile, the regular diet of the experimental mink had been withheld on the two days previous to the feeding to insure their eating the infected carcass. On August 3, 24 mink were fed average portions of infected meat mixed with a small amount of commercial mink cereal. As the taste of the mutton was naturally strange to them, each animal consumed approximately one-half the ration (3 oz.). All food was withheld on the following day to force the mink to eat the remaining food. On August 5, they were given the second and last feeding, and at this feeding, the animals consumed the food promptly.

The mink remained normal after both feedings and have never shown any symptoms suggestive of a Salmonella infection. At this writing, four months after the experimental feeding, 10 of the original 24 are alive and appear in excellent condition. The remaining 14 were used in other investigations.

Since no infection resulted from the feeding, and in order to determine if the mink were acting as carriers, the feces of each animal were collected on August 6, 9, 12, and 16. The method of isolation was carried out on each of the dates in a manner previously described. When possible, two typical colonies were picked from each Kauffmann plate.

On November 17, the feces of 10 of the original 24 mink were collected and cultured in order to determine if they were still acting as carriers. No Salmonella was found at this time.

Experiment 6.—Coinciding with the experimental feeding in experiment 5, infected meat from the same sheep was fed to a group of mink which had been previously given an anthelmintic.

TABLE 2—Results from Fecal Cultures Taken from Exposed Mink in Experiment 6

Bacteria	Number of colonies picked			
	Aug. 6	Aug. 9	Aug. 12	Aug. 16
Salmonella newport	12	10	10	11
Proteus spp.	0	0	0	0
Coliform spp.	0	0	0	1
Paracolon spp.	0	0	2	0
Total	12	10	12	12

It was believed that the infection was more likely to occur if there were some gastrointestinal irritation preceding the feeding; consequently, 9 young mink were given 0.2 cc. of

tetrachloroethylene on two occasions, five and two days before the experimental feeding. The regular diet was withheld, as in experiment 5, to make certain the infected meat would be eaten. This group of mink was fed the infected meat on August 3 and August 5. Their reactions were similar to those of the mink in experiment 5; *i.e.*, the taste of mutton was new to them. They were not fed on August 4 to insure their eating the test meat on the following day (August 5).

As in experiment 5, the mink in this group failed to become infected, and no symptoms were recorded. Five of the original 9 are alive and appear in excellent condition. The other 4 were used in other procedures.

As in the preceding experiment, the feces were cultured in an effort to determine if the animals in this group similarly acted as carriers.

Experiment 7.—The livers were removed from 2 guinea pigs which had died from a spontaneous infection of *S. typhimurium*. The tissue was ground and added to the regular ration of 2 young mink. No observable symptoms occurred, and they were alive four months after the feeding. No bacteriologic studies were made.

DISCUSSION

Penn[27] reported the isolation of members of the Salmonella group from mink in 14 per cent of all bacteriologic examinations. There is some question as to the validity of her work because of the high percentage of isolations and the possibility of confusion with organisms of the genus Proteus, a part of the mink's normal intestinal flora. No antigenic formulas or cultures were obtainable.

Some of the early isolations reported before the Kauffmann-White method of antigenic analysis came into use are somewhat open to question. It is possible that some of the reported isolations of Salmonella spp. were in reality members of the genus Proteus.

In most instances, other investigators have failed to produce paratyphoid infections in carnivores by oral exposure. Sherwood believed that cats possess an almost solid immunity to cultures of Salmonella given *per os*.[19] This seems unlikely, however, as there are authenticated isolations from cats exhibiting a gastroenteritis.[28,29]

It is not difficult to infect and cause the death of mink and ferrets by intraperitoneal injections of Salmonella, but this is not the natural method of exposure. Therefore, the greater part of the experimental work was concerned with adding virulent cultures to the diet.

Preliminary investigation in experiment 1 showed that normal animals were resistant to oral exposures. Since no infection occurred, it was thought that the lowering of resistance and subsequent inclusion of cultures in the food might result in infection.

The first depletion method employed was semistarvation, and only 2 animals among 12 became infected. Cultures fed at the same time to healthy animals given the regular diet caused no infection.

The second method used to lower the resistance of ferrets was to give them distemper, and when they first displayed symptoms of the virus disease, they were fed cultures. Large amounts of inoculum were added to the feed of ferrets having distemper, but there was no pathologic evidence of a secondary Salmonella infection.

To obtain material for histopathologic comparison, *S. newport* was inoculated intramuscularly into ferrets and mink. They are somewhat resistant to this method of introduction, as only 2 animals out of a total of 5 succumbed.

In experiment 2, the same animal should not have been used for delayed feeding, because of the possibility that the animal might develop an increased resistance to the organisms after the first trial. We were aware of this possibility, but as the animals are expensive and the supply of additional animals is limited, it was hoped the second exposures might cause an infection.

Since only 2 animals became infected by the feeding of broth cultures to 29 animals, the administration of organisms in broth does not seem to be a practical method of infecting ferrets and mink. It may be that the virulence of the organisms was reduced or destroyed in part by the acidity of the stomach. However, clear colonies that were urease-negative were isolated on SS agar plates; these were presumed to be Salmonella. Some of them may have been paracolon types. Accurate counts or analysis of these colonies were not made except in the experiments in part 2.

In order to more closely approximate a field outbreak, the animals in the experiments of part 2 were exposed by feeding infected carcasses. It was the purpose of these experiments to determine if the organisms were more likely to cause an infection when intimately distributed in a natural food. This method failed to produce an infection. Since the organisms were found in a large percentage of the fecal cultures, it appears that the animals acted only as carriers for a short time.

A Salmonella septicemia has been reported as occurring in the horse following the administration of oil of chenopodium,[30] and

Norwegian workers[31] have found that foxes may readily become infected with Salmonella after anthelmintic treatment. Administration of tetrachloroethylene failed to produce any increased susceptibility to subsequent *per os* exposure to Salmonella in mink.

SUMMARY

1) Intraperitoneal inoculations of *Salmonella enteritidis* in ferrets and mink will cause the death of the animal.

2) Mink and ferrets are somewhat resistant to intramuscular injections of *Salmonella newport*.

3) Infection with Salmonella organisms by oral administration was produced in only 2 animals out of 29. These 2 animals were in a group that received half-rations for an extended period.

4) Five ferrets given distemper and later fed cultures of *S. newport* showed no lesions of a secondary infection on postmortem examination.

5) Twenty-four young mink failed to become infected after the feeding of a sheep carcass which had been artificially infected with *S. newport*.

6) Nine mink, previously treated with an anthelmintic and fed the same material, also failed to become infected.

7) The livers, from 2 guinea pigs which died from a spontaneous infection of *Salmonella typhimurium*, fed to 2 mink did not cause an infection.

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